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BIOFLOCCULATION AND THE ACCUMULATION OF CHEMICALS BY FLOC-FORMING ORGANISMS

Patrick R. Dugan

Ohio State University

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BIOFLOCCULATION AND THE ACCUMULATION OF CHEMICALS BY FLOC-FORMING ORGANISMS

Municipal Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
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BIOFLOCCULATION AND THE ACCUMULATION OF CHEMICALS BY FLOC-FORMING ORGANISMS

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The Municipal Environmental Research Laboratory contributes to this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

Biological floc formation is an essential activity in the biological conversion of soluble organic waste material to insoluble settleable solids. A proper understanding of bioflocculation may lead to improvement in the activated sludge process. This report discusses the relationship of bioflocculation to waste treatment with special emphasis on the adsorption capacity of biopolymers for soluble and insoluble mineral and organic particles.

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ABSTRACT

Several floc-forming bacteria were isolated from polluted water by this and other laboratories. All organisms studied produced extracellular polymer fibrils that were related to flocculation. The extracellular polymers have high adsorption capacity for: soluble metal and other mineral ions, soluble organic nutrients (BOD), soluble toxic organics, insoluble mineral particles and insoluble organic particulates. The bacteria remove BOD by physical adsorption as well as by oxidative metabolism and can convert oxygen demanding organics to more extracellular polymer. Production of polymer can be stimulated nutritionally to yield amounts that have waste treatment-pollution abatement potential on a commercial scale. The relationship of bioflocculation to waste treatment and lake eutrophication is discussed and the basic mechanism of bioflocculation is considered. Biochemical activity of individual floc-forming cells is examined because of its relevance to polymer synthesis. Taxonomy of floc-formers is also considered in relationship to biochemical activities.

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SECTION I

CONCLUSIONS

The conclusions put forth are based upon the data and observations available from the limited number of organisms studied during the course of this investigation in addition to information available in the general literature. The general nature of some of the data remains to be established.

1. Bacterial floc-formation is related to the synthesis and presence of extracellular polymer fibrillar strands around individual cells. All floc-formers studied have been shown to produce extracellular polymer fibrils.

2. The chemical composition of extracellular polymer varies with bacterial species. The chemical composition and polymeric linkage of monometric units determines the physical and chemical properties of individual polymers and, therefore, determines their behavior in solution. That is, some polymers are more soluble than others, some react with specific ions to form complexes which readily settle from suspension, whereas others do not.

3. Excess carbon substrates are converted to extracellular polymer by the floc-formers. Polymer synthesis can be manipulated nutritionally. The polymer may be produced to such an extent and be of such a physical-chemical nature that it is recognized as a loose slime rather than a well-defined capsule around the cells.

4. Some polymers bind water to a greater extent than others. The more soluble extracellular polymers which become detached from cells and remain in colloidal suspension will result in increased viscosity of the surrounding medium when produced extensively by the cells. In waste treatment processes, this would have the net effect of reducing flocculation although it results from the factor that causes flocculation. This situation could be reversed by lowering the C:N ratio of input nutrients (sewage) or by addition of a chemical that will complex with the polymer strands.

5. Polymer can be produced in large amounts and this can be exploited as a means of removal of chemicals from solution providing the chemicals are of a type that form a complex with the polymer strands. There appears to be a high potential for removal of many cations from solution (e.g., transition and heavy metal ions) by this process. Secondary associations (e.g., anions to the cation-polymer complex) will allow extension of this procedure.

6. All of the polymers from the gram negative bacterial isolates studied are polysaccharides. These polymers have a high affinity for binding water. The polymer and associated water is a gelatinous mass from which the water is difficult to remove.

7. Presumably the water is organized in polarized ordered multilayers surrounding the polymer strands. This bound water appears to exchange with many metal ions. In so doing, a metal ion-polymer complex is formed which will precipitate (flocculate) from colloidal suspension and separate from the bulk solution.

8. It is postulated but not proven that water can be exchanged in two ways: (1) By chemicals in solution that have a greater affinity than the polymer for polarizing and orienting water in multilayers around them. In this way water would disorient from the polymer in favor of the ion or other chemical in solution. (2) By chemicals in solution that have a greater affinity for forming an association with the polymer structure than water does. This would result in a complex formation via exchange with bound water and would be similar to a polyelectrolyte or ion exchange process. In either case the physical properties of the polymer colloid would be altered and depending upon the ion or chemical associating with it, either flocculation or increased solubility could result.

9. In the case of a polymer matrix which surrounds cells, metal ions accumulate with the polymer and do not reach the cell membrane surface. This explains the high tolerance of such cells for ions that are normally toxic to cells.

10. With regard to #9 above we have also shown that organic nutrients (e.g., amino acids) are similarly adsorbed from solution and are oxidized by the cells later in time. This explains the high rate of BOD removal observed for floc-forming bacteria and also indicates that such cells can accumulate nutrients from an extremely dilute nutritional environment.

11. Insoluble particles such as clay, other bacteria, detritus, etc. can adsorb to polymer strands (See Fig. 46). This is referred to as formation of a conglomerate floc. It is a moot question as to whether particulates adsorb to polymer or polymer adsorbs to particle. Therefore, if the particle is of sufficient size the overall appearance will be adsorption of polymer to a surface or interface. In the latter case the polymer could be surrounding cells and then be attached to any suitable surface. For example, zoogloeal masses adhere to limestone surfaces in a trickling filter, encapsulated cells adhere to the walls of a flask or other container, polymer producing cells adhere to teeth and in general polymers collect at interfaces.

12. To a large extent the adsorption, concentration and accumulation of chemicals from solution via the mechanisms described are physio-chemical processes that are relatively non-specific by comparison to the high degree of specificity usually found in biological metabolic reactions.

13. Flocculation and floc-forming processes based upon the physio-chemical-biological interactions described are the same in waste treatment systems as they are in lakes. The differences between activated sludge removal of

oxygen demand and lake eutrophication is a matter of degree and complexity in so far as flocculation and its effects are concerned.

14. The gram negative floc-formers studied in this report all appear to be related taxonomically. That is, they cluster when analysed using numerical computer techniques and they share either identical or cross-reacting TCA soluble antigens. When compared to isolates obtained from other culture collections the organisms appear to be related to organisms which various investigators have identified as *Zoogloea*, *Pseudomonas*, *Acetomonas*, *Acetobacter* and *Gluconobacter*. Of course, many floc-formers have been observed which are known on the basis of other traits to belong to a variety of genera other than those named, including gram positive organisms.

15. Microbial production of polymers having ion adsorption and exchange capacities has potential for use in treating industrial waste water - particularly water contaminated with metal ions or radioactive minerals.

16. The reverse of the above conclusion (#15) has potential for controlling the chemical and physical properties of slime and biological floc in situations where slime is undesirable.

SECTION II

RECOMMENDATIONS

1. Pilot scale studies should be made for the purpose of scaling-up the polymer production-metal ion adsorption-flocculation process as a potential means of removing organic compounds and ions on a selective basis. For example, toxic metal ions or radioactive ions could be stripped from waste processing water prior to having it dumped into municipal sewage systems or other receiving water.

2. Adsorption to floc-former polymers of chemicals other than those examined have far reaching ramifications and should be studied. For example, enzyme adsorption has considerable potential with regard to sewage digestion and solid waste treatment. Health related implications in the area of tooth decay and diseases caused by encapsulated organisms (pneumonia, etc.) are undoubtedly related to the general phenomena described and should be investigated in that regard. Also the basic biological processes of mineral deposition such as in bone, teeth, shellfish, etc. are likely related to adsorption to polymers synthesized by cells and should be examined.

3. The high bound water content of activated sludge is a major problem and deterrant to its disposal. Means of separation of bound water from polymer in sludge should be studied and approaches to the problem are suggested by the conclusions of this report.

4. The generality of the conclusion of this report should be further established particularly as related to mixed cultures and environmental conditions (e.g., temperature and pH extremes) where practical abatement processes could be developed.

SECTION III

INTRODUCTION

The process of aerobic biological waste treatment is largely dependent upon two biological events: (A) Conversion of dissolved or suspended organic substances to microbial biomass via metabolic reactions. Since the organic substances are in a somewhat reduced chemical state, and therefore oxidizable, removal by metabolic conversion to cell substance represents biochemical oxygen demand (BOD) removal once the cells have been physically removed from the system. (B) The aggregation or flocculation of the biomass thus formed, results in settling and thereby provides a means for mechanically separating cells from supernatant (or in sanitary engineering terms, separating sludge from liquor). To a large extent, sewage or other organic wastes can be considered as microbial nutrients which are either in solution or suspended macro-molecules that are susceptible to attack by the hydrolytic enzymes produced by microbes. Flocculation affords a means of physically separating suspended particulates, including microorganisms and adsorbed substances, from the water phase. The suspended particulates found in waste water and sewage consist of colloidal dispersions of a variety of bacteria and other microorganisms in addition to non-living organic and inorganic substances. Flocculation is the aggregation or clumping of dispersed particles resulting in a floc which will settle from suspension upon standing, thereby leaving a clear supernatant.

The role of bacteria as the agents primarily responsible for flocculation as well as oxidation of reduced organic wastes in sewage treatment has long been recognized. Floc-formation appears to be related to the presence of capsular and/or slime polymers which are synthesized by bacteria and other microorganisms during the process of oxidation. In many instances the individual bacterial cells are embedded within a slime or capsular matrix which is often called a zoogloeal matrix. Some of the bacteria that produce rather characteristic zoogloeal matrices have been taxonomically classified as *Zoogloea*. Other bacteria which characteristically form flocs but differ in that they do not produce a typical zoogloeal matrix, have been identified as belonging to one of several other bacterial genera.

The biological phenomena described above as being basic to the success of aerobic BOD removal are complicated by adsorption on suspended particles of inorganics as well as organics that are not readily oxidized. Adsorption is a physical-chemical phenomenon but the biological formation of adsorption sites on sludge floc also relegates this to a consideration which overlaps biology and we will consider this aspect in more detail.

During the course of this investigation to examine the role of floc-forming microorganisms as biological agents for removal of BOD and toxic chemicals (metal ions, pesticides, etc.) from water, it became necessary to follow several divergent lines of research. It was considered essential to study (a) the process of flocculation; (b) the means or mechanisms by which these organisms exert a high rate of BOD removal; (c) mechanisms by which the bacteria either oxidize chemicals in solution or otherwise remove non-oxidized chemicals via complexing or absorption reactions; (d) examine in detail the structure and function of zoogloeal matrix material for its unique role in a, b, and c above; (e) study the process by which cells produce the zoogloeal matrix because of its potential as an abatement process for removal of selected chemicals from contaminated water and (f) attempt to clarify the confusing taxonomic status of the floc-forming bacteria in general and Zoogloea species particularly.

The general pattern of this report is to consider each of the above objectives (a through f) as separate phases which are discussed in context of pertinent related phases.

Research was initiated for the purpose of examining the functional role of the gelatinous matrix which surrounds, and is characteristic of the bacteria classified as belonging to the genus Zoogloea (10). No Zoogloea bacteria were available from culture collections or from laboratories which had reported on Zoogloea at the onset of this study. Bacteria were isolated from water samples and a strain was characterized and identified as Zoogloea according to published descriptions (9, 10, 14, 46, 100).

During our investigation into the nature of the zoogleal matrix a description of Zoogloea was published which stated that Zoogloea species do not possess a capsule or recognizable slime layer. On the basis of this description, the authors requested recognition of their cultures as the neotype of Z. ramigera Itzigsohn (29). This report necessitated a reappraisal of the identification of our isolates and prompted a more thorough study of the taxonomic status of Zoogloea in general. Reappraisal of cell morphology, particularly with reference to capsule or zoogleal matrix formation, led to an examination of literature pertaining to zoogleal-forming bacteria which are not generally recognized as belonging to the genus Zoogloea.

One objective of this report is to establish that an organism identified as Z. ramigera, based upon published descriptions, does possess a clearly recognizable gelatinous matrix. However, there appears to be no reason not to classify the same organism as a Siderocapsa species based upon published descriptions of this genus. The relationship of Zoogloea to gelatinous matrix-producing genera of the Siderocapsaceae is therefore considered. Further, the formation of a zoogleal matrix by a bacterial strain is not synonomous with floc formation since many species of bacteria are known to flocculate, but without synthesizing a gelatinous matrix (80).

Zoogloea ramigera is a gram-negative rod which appears to be a pseudomonad. It is characterized on the basis of its flocculent growth habit (14, 26, 33, 40, 93, 98) and a unique extracellular matrix which may be analogous to a capsule (14, 40, 93, 94). The isolate *Z. ramigera* 115 has been described as producing a zoogleal or capsular matrix, whereas another isolate, *Z. ramigera* I-16-M, has been described as having no matrix. Comparisons between these two isolates regarding flocculation and biochemical characteristics are reported in addition to comparisons to several other floc-forming pseudomonads (40). The extracellular zoogleal matrix has previously been examined for the purpose of elucidating its functional properties. The capacity of the extracellular polymer for concentrating metal ions from solution has been reported, and some of the ecological implications have been discussed (41).

The structure and composition of the zoogloeal matrix is considered in relationship to metabolic activities of the organism, which has been reported to possess an extremely active oxidative metabolism in the natural habitat (14, 16, 98).

Several bacteria have been isolated in this laboratory and elsewhere on the basis of their flocculent growth habit. Floc-forming bacteria grow primarily as clumps of large numbers of cells which may be surrounded by an extracellular polymer matrix (91). Because flocs settle out of suspension in the absence of agitation the medium rarely becomes turbid.

A distinction is made in this paper between "floc-formation" which implies growth in a clumped arrangement, and "flocculation" which implies aggregation from a turbid suspension after the cells have grown. This latter phenomenon is often referred to in the literature as "flocculation" (11, 12, 13, 23, 74, 83, 91) and is often an effect caused by the addition of chemical agents (e.g., blood sera, bentonite, polyvalent ions). On a physical-chemical basis the causes of flocculation and floc-formation may be similar with the differences being based on the origin and time involvement of the substance causing the clumping phenomenon.

Floc-forming bacteria are indigenous to natural water and the flocculent growth habit is exploited in biological waste treatment processes. Bacterial flocculation has been discussed by Crabtree et al. (28) and an explanation of the flocculation based upon induction by poly-beta-hydroxybutyric acid (PHB) storage granules has been proposed (26).

Tenney and Stumm (91) and Busch and Stumm (13) have described bioflocculation as "an agglomeration of cells resulting from specific adsorption of polymer segments and from bridging of polymers between cells." These authors presented chemical data based upon the ability of synthetic polyelectrolytes to cause aggregation of bacteria. They were able to show that polymers extracted from *Aerobacter aerogenes* behaved in the same manner as synthetic polyelectrolytes.

The Zoogloea ramigera strain 115 was shown to be composed of polysaccharide susceptible to cellulase and therefore believed to contain B-1-4, linkages (42). The polymer also possesses properties of a polyelectrolyte (40), as it binds high concentrations of metallic ions. Although the polymer is not readily observable around all Zoogloea isolates by techniques such as capsule stains, observation using electron microscopy revealed the presence of extracellular fibrils. These observations led us to examine several floc-forming bacterial isolates for the presence of extracellular polymer and to present microscopic evidence involving extracellular polymers as a cause of bacterial flocculation.

Extracellular matrix synthesis is, however, related to growth conditions and is, therefore, not an adequate taxonomic criterion (28, 40, 93). The fibrillar nature of the extracellular matrix of Z. ramigera 115 has been established (42). Polymer fibrils that resemble cellulose have been observed around additional Zoogloea isolates as well as non-Zoogloea floc-forming bacteria (43). This has led to the consideration of possible taxonomic relationships among the so-called floc-forming Pseudomonadaceae and Zoogloea to recognized species of Pseudomonas and Acetobacter.

There are few reports concerning antigenic relationships among the Pseudomonadaceae other than for the genus Pseudomonas. An exception is the work of McIntosh (63) in which an antigenic analysis of various Acetobacter species was made using agglutination and tube precipitation tests. He reported that the A. suboxydans strains constituted a distinguishable serologic group and that A. rancens strains also exhibited serologic relationships, but were unrelated to A. suboxydans. A. oxydans, A. xylinum, and A. melanogenin were not included in the study.

This report is intended to demonstrate and clarify: (i) antigenic relationships among similar isolates which produce cellulase susceptible extracellular polymers, (ii) to compare them to type species of Pseudomonas and Acetobacter, and (iii) to establish the relationship of isolates classified as Zoogloea to Pseudomonas and Acetobacter.

Antigenic relationships of the four isolates were examined by immunodiffusion and were compared to three cultures obtained from American Type Culture Collection. The type cultures were selected on the basis of similarity of substrate range, particularly alcohol metabolism, and because Acetobacter is the only pseudomonad reported to synthesize cellulose which is of course a cellulase susceptible extracellular polymer.

Biological waste treatment processes convert reduced organic substances to microbial cell mass. The energy required to drive this synthetic process is derived from oxidative reactions catalyzed by the enzyme systems of the microorganisms. Once the organic material has been converted to microbial cells, biological flocculation becomes an essential part of the process because it forms the basis for removal of partially oxidized material from the water. Relatively little is known with regard to the role of dissolved minerals in the treatment process, particularly in relation to growth of microorganisms. Current opinion is that mineral content as well as organic content of treated waste plays an important role in stimulating growth of algae and other plant forms in receiving waters (36, 39).

Our interest in *Zoogloea* species concerns the ability of the organism to concentrate and accumulate dissolved chemicals from solution. We are also attempting to elucidate the functional role of the gelatinous matrix in the metabolic activities of the organism. This study is stimulated by potential application in the following areas: (i) as a means for selective removal of minerals and/or organics by waste treatment processes resulting in a retarding influence on the eutrophication process in receiving waters, (ii) recovery of isotope contaminants from aquatic environments, (iii) basic information concerning nutritional requirements and environmental influences on activity of the organisms will allow more efficient design of specialized aerobic treatment facilities.

The gelatinous matrix producing *Zoogloea ramigera* strain 115 (40) and the nongelatinous matrix producing strain I-16-M of Crabtree et al. (26, 28) were studied with respect to metal ion uptake.

After adsorption of individual ion pairs from solutions was examined, an evaluation of a complex mixture of ions from a natural source was studied. Samples of acid mine drainage were selected for this purpose.

Acid mine drainage is characterized by high concentrations of dissolved iron, sulfate and hydrogen ions, as well as various other ions in lower concentrations. It is the adverse influence of these ions both in drainage streams and in receiving water that elicits the water pollutional stigma so often associated with mining operations.

My colleagues and I have argued that the most sensible approach to control of mine drainage pollution would be to prevent its formation at the source. One specific suggestion was to selectively inhibit the acidophilic autotrophic bacteria (*Thiobacillus*-*Ferrobacillus* group). These organisms are responsible for production of much of the sulfate and ferric iron ions via metabolic oxidation of pyrite and related minerals. They are indirectly responsible for a considerable amount of acid formation because the ferric ion they produce, reacts with water yielding "yellowboy" and acid (H^+).

Treatment of the problem, once formed, is then a "catch up" procedure and acid pollution abatement would be a continuous effort. Although treatment is not an ultimate solution to the problem, situations do arise where immediate action is demanded and practical means of prevention are not yet available. We take the viewpoint that treatment is essential while adequate preventative measures are being sought, and have suggested the utilization of sulfate reducing bacteria as having potential in this regard. Anaerobic sulfate reducing bacteria in the *Desulfovibrio* and *Desulfotomaculum* genera have the demonstrated capacity to reduce sulfate to sulfide with concommitant increase in pH and removal of iron ions by precipitation as black $\underline{FeS}$.

The present suggestion represents another potential microbiological treatment process for removal of mine water ions. The data herein pertain to removal of ions found in acid mine drainage by adsorption to extracellular polymers that are synthesized by microorganisms under their natural growth conditions. In this context I would like to emphasize that this is analogous to the processes now in use for treatment of municipal and domestic wastes. Virtually all such processes in current use are biological processes which depend upon the metabolic activity of microorganisms. The point to be made is that much of the engineering technology is already available and could be extrapolated for use in abatement of acid mine drainage pollution.

Many chlorinated hydrocarbon insecticides have been isolated from surface waters, usually in concentrations of less than 1 μg/liter. Significance of the presence of pesticides in low concentrations has been established (25), for example, with reference to accumulation in shellfish and also in regard to chronic synergistic influences on fish.

Certain questions arise. For example, how do highly insoluble chlorinated hydrocarbons enter the aquatic system and once there, how are they transported? Organic pesticides can enter the ground and surface waters in a number of ways: by direct application for control of aquatic weeds, trash fish, and aquatic insects; from discharge of industrial waste water; by runoff from agricultural land; by drift from aerial and land applications: from clean-up of equipment used for pesticide application; and finally, by accident.

Our interest lies in the fate of these chemicals once in the water column, and particularly, in their adsorption to silt and floc-forming bacteria which form contemporary sediment in lakes.

In this context, the adsorption of the chlorinated hydrocarbon pesticide, aldrin, by floc-forming bacteria and lake silt was selected as an experimental system, although any one of a number of other organic pesticides could have been used.

A preliminary study of aerobic heterotropic bacteria isolated from Lake Erie indicated that of thirty-eight bacteria isolated and examined, fourteen formed flocs in at least one of the six different growth media tested, one of which was sterile lake water. Two of the isolates which formed flocs in sterile lake water were selected for further study with regard to their ability to concentrate and accumulate the pesticide aldrin from solution. One bacterium was an orange-red pigmented gram negative (G-) pseudomonad rod. The other was a gram positive (G+) Bacillus species.

Once it had been demonstrated that floc-forming bacteria were ubiquitous in nature and that the polymer produced by the floc-formers would adsorb a wide variety of chemical substances (anions, cations, soluble and insoluble organics, pesticides, inorganic and organic insoluble particulates), it was of interest to investigate the capacity of cells for producing extracellular polymer. Further investigations of polymer formation, organic oxidation and the influence of growth conditions on polymer production ensued, with the ultimate objective of evaluating the potential for large scale polymer production and its use for water treatment on a large scale.

SECTION IV

MATERIALS AND METHODS

Organisms

Zoogloea ramigera 115 and *Pseudomonas* species C-3 were isolated from a trickling filter at this laboratory and have been previously described (40, 42, 43). *Z. filipendula* P-8-4, *Z. ramigera* I-16-M, *P. denitrificans* P-95-5 and an unidentified gram negative rod C-22-4 were also isolated from sewage by Crabtree et al. (40) and considered elsewhere (40, 42, 43). *P. denitrificans* (ATCC 13867), *Acetobacter oxydans* (ATCC 6433), and *A. suboxydans* (ATCC 621) were obtained from the American Type Culture Collection. *Z. ramigera* Z-SC-38 was isolated by Ganapati et al. (44).

Isolation of Organisms

Isolations were made from limestone covered with zoogleal masses and effluents taken from organically polluted water. Material on the stone was scraped off and placed into 0.05% proteose-peptone yeast extract broth (PPYE, Difco). The gelatinous material was disrupted by exposing small clumps to ultrasound (Branson, 20Kc output) in sterile PPYE until the clumps dispersed. Samples were serially diluted in PPYE and incubated at 28°C. After three days the highest dilutions containing growth (10^{-7} to 10^{-10}) were streaked onto PPYE agar and Tryptone Glucose Extract Agar (TGE, Difco). When sufficient growth occurred, colonies of differing morphological types were transferred to PPYE in duplicate. One tube was placed in an incubator at 28°C and the other was placed on a rotary shaker (60 rev/min) at 24° ± 1°C. Incubation was continued until growth was observed. Those tubes containing a pellicle (stationary) or a floc (shaken) were selected and the entire isolation procedure was repeated until pure cultures were obtained.

Characterization of Organisms

Cells were inoculated into 100 ml of 0.05% PPYE or 0.5% Trypticase Soy Broth (BBL) and grown on a rotary shaker at 24°C ± 1°C for 48 hours. They were washed twice with 0.3M phosphate buffer and resuspended in 10 ml of buffer.

Casein Hydrolysate (0.2% enzymatic, Difco) containing 16 μg/ml brom thymol blue indicator served as the basal medium for carbohydrate utilization studies. Carbohydrates were added to the basal medium to give either 1% (w/v) solutions or as differential discs (1 disc/7 ml, Difco). Sugars other than discs were sterilized by autoclave. Occasionally a slight acid reaction was detected as the result of autoclaving; in these cases the medium was sterilized by membrane filtration. Alcohols were prepared in 1% concentrations. Crystal violet was added to TGE to give a final concentration of 0.001% for use as a differential growth medium. All other media were prepared according to standard methods (87). Tubes were inoculated with 0.1 ml of a cell suspension and results were read after 24, 48, and 120 hours.

Growth of three isolates designed as 115, C-3, and I-16-M was examined in various defined growth media for the purpose of comparing flocculation, zoogloea matrix formation, and growth. The media examined were: (A) the arginine medium of Crabtree et al. (27). This medium was varied by adding (A_1) folic acid, 10 mμg/ml; or (A_2) folic acid, 10 mμg/ml plus glucose, 0.5%, (B) the ammonium nitrate medium of Crabtree (Ph.D. thesis, Univ. of Wisconsin, Madison, 1966) NH_4NO_3, 0.1%; $MgSO_4 \cdot 7HOH$, 0.02%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; and glucose, 0.5%. This medium was varied by adding either (B_1) vitamin B_{12}, 1.5 mμg/ml; or (B_2) B_{12}, 1.5 mμg/ml plus folic acid, 10 mμg/ml plus biotin, 2.0 mμg/ml. (C) the casamino acid medium of Crabtree (Ph.D. thesis, Univ. of Wisconsin, Madison, 1966), which contained vitamin free casamino acids 0.1% (Difco); $MgSO_4 \cdot 7HOH$, 0.02%; K_2HPO_4, 0.2%; KH_2PO_4, 0.1%; B_{12}, 1.5 mμg/ml; biotin, 2.0 mμg/ml. This medium was varied by adding either (C_1) folic acid, 10 mμg/ml or (C_2) folic acid, 10 mμg/ml plus glucose, 0.5%.

Because of time and manpower constraints all isolates examined could not be studied in equal detail within the scope of the experimental objectives. Therefore, greater attention was given to Zoogloea ramigera 115 than to other isolates due to interest in the uniqueness of the organism as a zoogloeal matrix producer. Z. ramigera 115, in addition to a closely related isolate, Z. ramigera 1, were further characterized biochemically and nutritionally in an attempt to assess their role as agents for BOD removal in waste treatment processes.

The isolates have the following general descriptions after 72 hours cultivation at 25°C on PPYE medium which contained 0.001% crystal violet (either broth or 1.5% agar).

Isolate 1:	Gram-negative, freely motile rods, 0.5μ long; sudanophilic granules present. Colonies were straw-colored and leathery to needle touch, round with an entire margin. No ridges or convolutions were present. Crystal violet was decolorized.
Isolate 115:	Gram-negative rods, 1μ long; sudanophilic granules present; cells held together in a floc or matrix but swam away from the finger-like projections of the matrix. Colonies were circular with a slightly scalloped or convoluted margin, leathery to the touch and difficult to break up. Crystal violet was decolorized.

Culture Conditions

The following basal growth media were used for studies on substrate utilization in addition to standard differential media (87).

Basal medium I (Dugan and Lundgren, 1960)

KH_2PO_4	2.5 g	$CuSO_4 \cdot 5H_2O$	0.1 g
K_2HPO_4	1.25 g	$FeSO_4 \cdot 7H_2O$	0.1 g
$MgSO_4$	1.0 g	$MnSO_4 \cdot H_2O$	0.1 g
Na_2MoO_4	0.1 g	$ZnSO_4 \cdot 7H_2O$	0.1 g
Boric Acid[1]	0.1 g	$CaCl_2 \cdot 2H_2O$	0.1 g
$Co(NO_3)_2 \cdot 6H_2O$	0.1 g		
Double distilled deionized H_2O			1000.0 ml

Basal medium II (Crabtree et al., 1966)

$MgSO_4 \cdot 7H_2O$	0.2 g
K_2HPO_4	2.0 g
KH_2PO_4	1.0 g
Distilled H_2O	1000.0 ml

Basal medium III (for primary carbon sources)

Casamino acids, vitamin free	3.0 g
Vitamin B_{12}	1.5×10^{-4} g
Basal medium I	1000.0 ml

Basal medium IV (for primary carbon sources)

Yeast extract	0.5 g
Basal medium III minus B_{12}	1000.0 ml

Basal medium V (for primary carbon sources)

Biotin	0.01 g
Folic acid	0.01 g
Thiamine	1.0 g
Basal medium III minus B_{12}	1000.0 ml

Basal medium VI (for sole carbon sources)

$(NH_4)_2SO_4$	0.5 g
Biotin	0.01 g
Folic acid	0.01 g
Thiamine	1.0 g
Basal medium I	1000.0 ml

Proteose Peptone-Yeast Extract (PPYE) medium

Proteose Peptone	0.5 g
Yeast Extract	0.5 g
Distilled H_2O	1000.0 ml

Chitin and Collagen Media

Basal medium I, supplemented with yeast extract (5 mg/ml), was employed in the examination of isolates 1 and 115 for the presence of extracellular chitinase and collagenase. Chitin or collagen, in 10% (w/v) concentrations, were dispersed in distilled water in a Waring Blendor. These suspensions were sterilized separately from the basal medium by autoclaving at 121°C for 10 min. They were then added aseptically to the liquid medium for Erlenmeyer flask shake cultures (New Brunswick platform shaker, 160 rpm) and to solidified plating medium, to give a final concentration of 1 mg/ml.

Carbon and Nitrogen Sources

Basal medium II was employed in conjunction with various nitrogen and carbon sources, supplemented with the following growth factors: (A) vitamin B_{12} (1.5×10^{-7} μg/ml); (B) folic acid (0.01 μg/ml), biotin (0.01 μg/ml), thiamine (1.0 μg/ml); (C) Yeast Extract (10 μg/ml). (This concentration did not supply sufficient carbon and nitrogen to support growth.) pH was adjusted to 7.0 using either 2N HCl or 2N KOH.

Ammonium salts. NH_4NO_3, $(NH_4)_2SO_4$, and NH_4Cl were tested as nitrogen sources in basal media I and II, at 0.5 mg/ml concentration. The three growth factor systems mentioned above were studied using the following concentrations of glucose as carbon source: 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.5 mg/ml, 5.0 mg/ml, and 10.0 mg/ml.

Amino acids. The examination of 20 amino acids as sole carbon and nitrogen sources was carried out in basal media I and II, with vitamin B_{12} and yeast extract as growth factors. The basal media were supplemented with glucose (20 mg/ml) when it was desired to determine the ability of the organism to use the amino acids as a source of nitrogen only. Amino acids were supplied in concentrations of 1.5×10^{-4}M, 1.5×10^{-3}M, and 1.5×10^{-2}M.

These same basal media, both with and without the amino acids, were supplemented with guanine, cytosine, and adenylic acid, individually and as a mixture to give a final concentration of 70 μg of each nitrogen base per milliliter of medium. Stationary and aeration tube cultures were used in this study.

Sugars and sugar alcohols. The ability of isolate 115 to utilize numerous sugars and sugar alcohols as primary and sole carbon sources was examined in basal media II (containing either arginine (0.5 mg/ml) or $(NH_4)_2SO_4$ (0.5 mg/ml), III, IV, V, and VI. All sugars were filter sterilized (Millipore, 0.45 μ pore size) and added to the basal media (50.0 mg/ml) in Erlenmeyer flasks and incubated at both 24°C and 30°C on a platform rotary shaker. The cultures were examined for changes in pH with a Corning expanded scale pH meter (Corning Instrument Co., Corning, N.Y.) at 12-hr intervals for 5 days.

Organic acids and alcohols. Several organic acids and alcohols were examined as sole and supplementary carbon sources. It was assumed that concentrated methyl, ethyl, propyl, isobutyl, sec-butyl, n-amyl, dodecyl, lauryl, and stearyl alcohols would not contain living contaminants, and the alcohols were added aseptically directly to sterile basal media. Formic, acetic, propionic, butyric, alpha-ketobutyric, beta-hydroxbutyric, caproic, and alpha-ketoglutaric acids were filter sterilized. Capric, lauric, and stearic acids were autoclaved before addition to the basal media. The basal media used were I and II, supplemented with arginine or $(NH_4)_2SO_4$, and PPYE. The final concentrations of acids or alcohols in the media were 0.5%, 1.0%, and 2.0% (liquids, v/v; solids, w/v). The studies were carried out in shaken Erlenmeyer flasks, aeration tubes, and on agar plates. Homogenous media were not obtained with C_{12} and C_{18} acids and alcohols.

Preparation of Antigens

For the immunization of rabbits, antigens were prepared as follows. *A. oxydans* was grown in 0.5% Tryptone Glucose Extract broth (TGE, Difco) at 26°C for 24 hours. All other organisms were grown in 0.05% Trypticase Soy Broth (TSB, Difco) under the same conditions. The cells were washed twice with 0.85% saline, resuspended in 20 ml of saline, treated with ultrasound for 1/2 to 3/4 minute with a Bronwill Biosonik III Sonicator (Bronwill Co., Rochester, N. Y.) to disrupt the floc and polymer, and diluted with saline to correspond to a nephelometer tube calibrated to 3×10^8 cells/ml.

For the test antigens, cultures were grown for two to three days at 26°C in 12-liter fermenters containing either 0.05% TSB or 0.5% TGE. The cells were collected with a Sorvall RC2B Superspeed Centrifuge, using the KSB continuous flow apparatus (Ivan Sorvall, Inc., Norwalk, Conn.). A modification of the trichloroacetic acid TCA extraction method of Van Eeden (96) was employed to prepare the bacterial antigens used in serologic tests, the TCA soluble extracts were dialyzed at 4°C against three changes of phosphate-buffered saline (pH 7.3), instead of a water-thiomersalate mixture. An extract of 0.005% TSB was prepared in the same way, as a control. The extracts were stored at 4°C after the addition of 0.02% sodium azide, as a preservative.

Gel Diffusion Tests

A modification of Campbell's (17) method was employed. Seventy-five milliliters of 0.85% saline and 15 ml of double-distilled water were added to 0.85 gm of Special Agar Noble (Difco). The mixture was autoclaved for 15 minutes, allowed to cool to 55°C, and 10 ml of 1:1000 stainless merthiolate (Eli Lilly Co., Indianapolis, Ind.) and 2.5 ml of 0.1% aqueous trypan blue was added to the cooled agar. Five millimeter wells were cut 9 mm from center to center in a 3.0 mm agar bed in Petri dishes, and 0.05 ml of reactant was added to each well. The plates were incubated at 37°C for 24-36 hours. Sera were concentrated by a factor of 2 or 3 with a Diaflo Model 50 ultrafiltration cell containing a UM 10 membrane (Amicon Corp., Lexington, Mass.). The TCA-extracted antigens were concentrated by a factor of 4 or 16 by pervaporation with subsequent dialysis against buffered saline (pH 7.3). After testing each antigen with each serum in hexagonal well patterns, all indications of possible relationships were re-examined separately in adjacent wells. Inconclusive reactions were aided by further concentrating the antisera and antigens involved.

Nucleic Acid Base Ratio Determination

Deoxyribonucleic acid (DNA) was extracted by a modification of the Marmur procedure (62). Because of the large amount of polysaccharide present it was necessary to homogenize the samples in the presence of sodium lauryl sulfate. Polymer was precipitated by adding isopropyl alcohol in place of ethyl alcohol during the initial DNA precipitation before proceeding with the deproteinization step. In many cases it was not necessary to treat with RNase after deproteinization because DNA was selectively precipitated during early steps in the procedure. It was assumed that the large amount of polysaccharide adsorbed salts from solution which thus altered the delicate salt balance that controls precipitation of the DNA.

DNA base composition (% G-C) was determined by the buoyant density method of Shilderkraut et al. (84). The DNA-CsCl solution was centrifuged in a Beckman model E analytical ultracentrifuge (AnD rotor) at 40,000 rpm and 23°C. Photographs of the density gradient were taken at 4 hr. 16 min. intervals.

Microscopy of Cells

(A) *Cultures*. Cells were grown in a modified version of Crabtree's (27) arginine salts medium which contained per 100 ml: arginine·HCl, 0.05 g; $MgSO_4 \cdot 7HOH$, 0.02 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1 g; glucose, 0.5 g; B_{12}, 1.5×10^{-7} g. Cultures were shaken at 28°C for 72 hours.

Brightfield and ultraviolet microscopy. A 0.1% aqueous solution of Paper White-BP (a diamine stilbene disulfonic acid dye which has a high affinity for cellulose; E. I. DuPont de Nemours and Co. Inc., Chicago), was used to stain 72 hr cultures of *Z. ramigera* 115 and *Z. ramigera* I-16-M in a manner similar to that described by Harrington and Raper (47). The stained cell flocs were examined by both brightfield and ultraviolet microscopy. Photomicrographs were taken on Kodak Plus-X film. Exposure times were 5 to 8 sec in visible light and 15-90 sec in ultraviolet light.

(B) *Preparation for electron microscopy*. *Z. ramigera* isolates 115 and I-16-M were grown in a modification of the arginine salts medium of Crabtree (28) which has the following composition per 100 ml: arginine·HCl, 0.05 g; $MgSO_4 \cdot 7HOH$, 0.02 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1 g; glucose, 0.5 g;

B_{12}, 1.5 x 10^{-7} g. Proteose-Peptone yeast extract, 0.05% (PPYE, Difco) was used to obtain flocculent growth of cultures other than *Z. ramigera* 115 and *Z. ramigera* I-16-M. The bacteria were cultured in Erlenmeyer flasks containing 100 ml of growth medium and were either incubated on a reciprocal shaker (100 strokes/min) at 28°C until flocculent growth occurred, or held stationary at 28°C until a pellicle formed. Flocs or pellicles were washed twice with distilled water. In some experiments this was followed by boiling the cell-flocs in 1 N NaOH for 20 min. (72) or by allowing them to stand for 24 hours at 28°C to remove alkali-soluble material.

(C) *Shadow-casting and carbon replicas*. The cell-flocs were placed on Formvar coated grids and shadowed with carbon platinum or they were shadowed and then carbon coated. Carbon replicas were prepared by the technique of Bradley and Williams (8).

(D) *Freeze-etching*. The flocs were centrifuged into a pellet and a portion of this was placed on a small (3 mm) copper disc, previously scratched to insure greater adherence. The disc was immediately frozen in liquid Freon 22 and transferred to liquid nitrogen. The object was then placed on a pre-cooled (-150°C) table in a Balzers apparatus (model BA 360 M; Balzers, Principality of Leichtenstein) and frozen-etched as described by Moor and Muhlethaler (67) and Moor (66). After freeze-etching, the discs containing the specimens were removed, warmed to room temperature, and dipped into distilled water to remove replicas from specimens. To remove cells which remained attached to replicas, treatments of 70% sulfuric acid (1 hr.), distilled water rinse, Eau de Javell (a commercial bleaching agent containing 14% NaHOCl in NaOH), and final distilled water rinses were employed. After the replicas were picked up on Formvar-coated grids and allowed to dry, they were ready for examination in the electron microscope.

(E) *Negative staining*. The cell pellets from centrifuged flocs were washed twice with sterile distilled water and a portion was placed on a carbon-coated Formvar grid. This was stained with 0.5% phosphotungstic acid (pH 7.0) and examined in an electron microscope (50KV).

(F) *Thin section preparation*. Flocs were fixed in 1.0% OsO_4 in veronal buffer overnight in a refrigerator and stained in uranyl acetate for 2 hours. Dehydration of the flocs was performed with a linear gradient dehydration device (78) using ethyl alcohol. Each floc was embedded in Epon 812 monomer according to the method of Luft (61) and polymerized by heat. Sections were cut on a Porter-Blum MT-1 Ultra Microtome (Ivan Sorvall, Inc., Norwalk, Conn.) using glass knives (Ferval Co.) and post stained with lead citrate.

(G) *Bovine serum albumin for phase contrast microscopy*. A 42% solution of bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared and mixed with cell suspension (1:1) on a glass slide for the purpose of increasing the refractive index of the suspending fluid. The wet mount was examined using phase-contrast microscopy (2). Cells were stained for light microscopy as previously reported (40).

Purification of extracellular polymer from culture 115 cells. The cell-floc matrix was separated from culture supernatant fluid by centrifugation at 10,000 x g for 20 minutes and the supernatant fraction was discarded. The floc pellet was washed once with distilled water, centrifuged, and suspended in distilled water (1 g wet wt. cells/7 ml water). The suspension was exposed to ultrasound (Branson Sonifier, 20 KC output at 3.0 amp. for 5 min.).

The efficiency of matrix removal from cells was determined by examining the suspension periodically in a phase-contrast microscope using wet mounts containing cells suspended in 0.03% crystal violet. The ultrasound treated suspension was centrifuged at 8000 x g for 10 minutes to remove cells and debris. The viscous supernatant fraction was saved, added to 5 volumes of methyl alcohol and stored at -20°C overnight. A white flocculent precipitate rose to the surface upon standing. It was removed with the aid of a watch glass and was air-dried to remove aqueous-methyl alcohol. The precipitate was dissolved in distilled water at 60°C with agitation and precipitated in 5 volumes of methyl alcohol. The precipitate was recrystallized 2 times using the above procedure and air-dried. The dry material was dissolved in 2 N NH_4OH (approximately 100 μg/5 ml), and centrifuged at 40,000 x g for 3 hours to remove material insoluble in NH_4OH. The water clear supernatant fraction containing dissolved polymer was added to an equal volume of 95% ethyl alcohol. As the polymer precipitated from the solution it was removed by means of a glass rod and added directly to a carbon-coated grid for examination in the electron microscope.

Infrared spectroscopy of purified polymer. The purified polymer was also added directly to a NaCl crystal under constant exposure to a heat lamp to facilitate rapid solvent evaporation. The NaCl crystal which was coated with a thin film of the polymer was examined using a Perkin Elmer Model 237B Spectrophotometer.

Enzyme susceptibility of purified polymer. Alpha-amylase (*Bacillus subtilus*, type IIA, Sigma Chem. Co., St. Louis, Mo.) and beta-amylase (barley, type IIB, Sigma Chem. Co.) were added separately to purified polymer and hydrolytic activity was assayed according to the procedures described by Bernfeld (5).

One ml of an alpha-amylase solution (0.03 mg/ml) was added to 1.0 ml of polymer solution (0.36 mg/ml of 0.02M NaH_2PO_4 - 0.006M NaCl at pH 6.9) and replicas were incubated at 20°C for time intervals up to 120 minutes.

One ml of beta-amylase (0.06 mg/ml) was added to 1.0 ml of polymer solution (0.36 mg/ml of 0.016M sodium acetate, pH 4.8) and replicas were incubated at 20°C for time intervals up to 120 minutes.

One ml of soluble starch (10 mg/ml) was added to both alpha and beta-amylase solutions in place of polymer to serve as a positive control.

The assays were repeated several times using whole isolate 115 cell-flocs and I-16-M cell-flocs in place of purified polymer.

Cellulase (*Aspergillus niger*, type II, Sigma Chem. Co., 30 mg/15 ml of 0.05M sodium citrate, pH 4.0) was added to whole isolate 115 cell-flocs or I-16-M cell-flocs and incubated at 24° ± 1°C for periods up to 120 minutes. At desired time intervals, 1.0 ml of Nelson's alkaline copper reagent was added to each tube and the solution was assayed colorimetrically for sugar as described by Nelson (70). Carboxymethylcellulose (12.0 mg/ml) was added to buffered enzyme in place of cells and served as a positive control.

Ester formation. Cultures of *Z. ramigera* isolate No. 1 and No. 115 were maintained on slants of 0.5% (w/v) Proteose Peptone (Difco, Detroit, Mich.), 0.5% (w/v) Yeast Extract (Difco) solidified with 1.5% agar (Difco) at 28°C and in Proteose Peptone-Yeast Extract broth at -20°C.

Short chain alcohols, C_1 through C_8 (including primary and secondary butyl alcohol), stearyl, and lauryl alcohols were tested in concentrations ranging from 0.1% to 10% (v/v) as substrates for ester production and as sole carbon sources. Culture media ingredients per liter were: (A) Proteose Peptone (Difco), 0.5 g; Yeast extract (Difco), 0.5 g; ethyl alcohol (95%), 23.0 ml. (B) arginine·HCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; K_2HPO_4, 2.0 g; KH_2PO_4, 1.0 g; vitamin B_{12}, 1.5×10^{-7} g; ethyl alcohol, 23.0 ml. (C) $(NH_4)_2SO_4$, 0.5 g; $MgSo_4 \cdot 7HOH$, 2.0 g; K_2HPO_4, 2.0 g; KH_2PO_4, 1.0 g; B_{12}; 1.5×10^{-7} g; ethyl alcohol, 23.0 ml.

Ester production in liquid media was investigated in Erlenmeyer flasks, containing media in volumes equal to 50% of the flasks' capacities, both as stationary and shaken (gyratory shaker, 160 rpm, New Brunswick Scientific Co., New Brunswick, N. J.) cultures and in 8 L. of medium in an aerated fermentor (New Brunswick Scientific Co.). Ester production was also examined in agar plate cultures incubated at temperatures in the range of 4°C to 45°C in a gradient temperature incubator (Lennox Corporation, Columbus, Ohio).

Shaken cultures were sampled several times during growth for the production of ester and assayed by the two-dimensional thin-layer chromatography technique of Bleiweiss (6) for intermediary acid compounds. One-dimensional paper chromatography (Absolute ethyl alcohol:ammonium:hydroxide:water (160:10:30)) was also employed in the assay of intermediates; acid spots were located with bromphenol blue spray (0.04% w/v alcoholic bromphenol blue). Microorganic spot tests (Feigle, 37) were employed to detect esters and intermediate acids. The presence of poly-beta-hydroxybutyric acid, reported to be present in this organism (Parsons and Dugan, 71) was determined by light and phase microscopy.

Ester in liquid cultures was detected and identified by a combination of infrared spectroscopy and gas-liquid chromatography (GLC). Five GLC column packings were tested: 10 ft. Carbowax 600 (10% w/w) on Chromosorb W (Varian Aerograph, Walnut Hills, Calif.), 10 ft. Carbowax 1540 (15% w/w) on Chromosorb W (Varian Aerograph), 3 ft. Hercoflex 5% on Chromosorb W (Varian Aerograph), 23 ft. column composed of the latter three columns in sequence, and 5 ft. ethylene glycol succinate (15% w/w) on Gas-Chrom P (Applied Science, State College, Pa.). Nitrogen, with a flow rate of 25 ml/min at the exit port was employed as the carrier gas. Injector and detector temperatures were 165°C. The column was operated isothermally at 45°C for ester identification. A linear program with a rise of 4°C/min to 145°C was employed for examination of other culture products. Glass columns (I.D. 1/8 in) were used in an Aerograph model 204 gas-liquid chromatograph, equipped with a hydrogen flame detector (Varian Aerograph).

Figure 1 summarizes the procedures followed in concentrating and identifying the ester produced by the culture. Cell-free culture supernatant fluids were directly injected into the GLC column or concentrated before injection by one of two methods: (A) the cell-free supernate was placed in a dialysis tube and concentrated by water removal with Aquacide II (Cal Biochem). (B) the cell-free supernate was continuously extracted in a counter-current continuous extracter using ethyl ether (anhydrous).

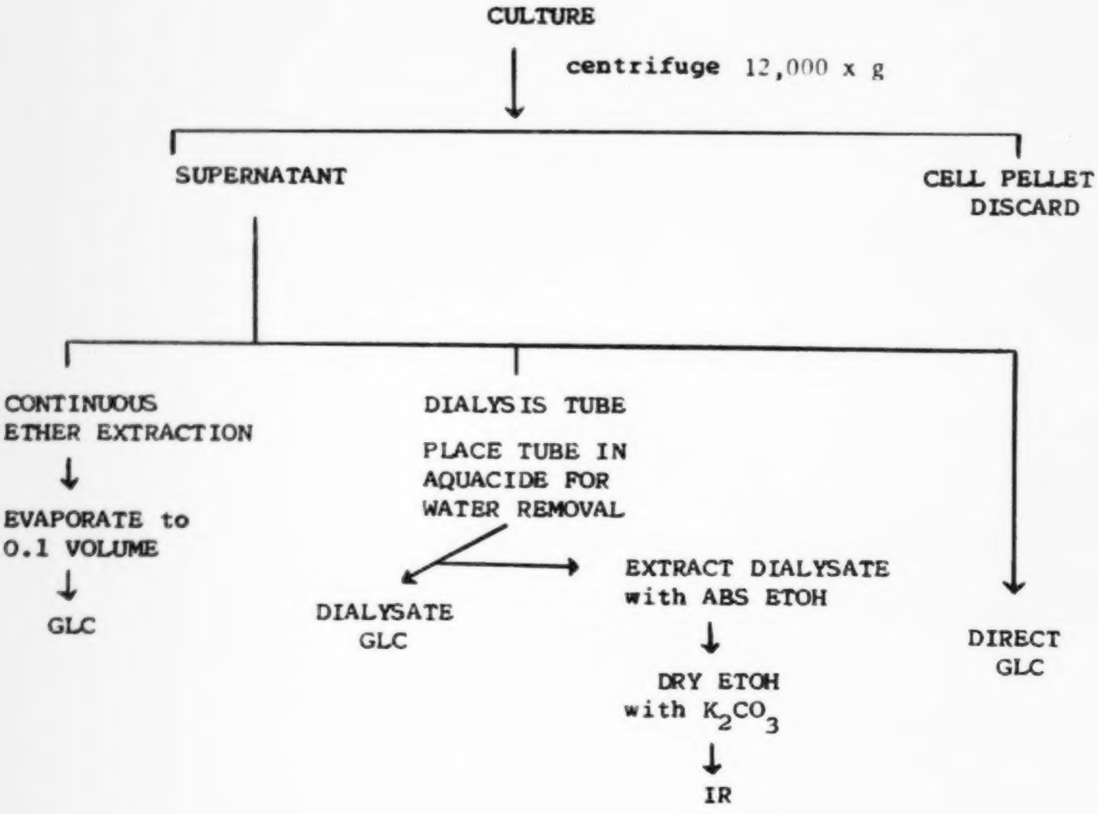

CULTURE
centrifuge 12,000 x g
SUPERNATANT
CELL PELLET DISCARD
CONTINUOUS ETHER EXTRACTION
EVAPORATE to 0.1 VOLUME
GLC
DIALYSIS TUBE
PLACE TUBE IN AQUACIDE FOR WATER REMOVAL
DIALYSATE GLC
EXTRACT DIALYSATE with ABS ETOH
DRY ETOH with K_2CO_3
IR
DIRECT GLC

Figure 1. Flow diagram for ester concentration and identification

Cells in mid-log growth stage, grown in the presence and absence of ethyl alcohol, and their cell-free culture supernatant fluids were employed in assays to determine the existence of an esterifying enzyme, either associated with the cells or free in the medium. The procedures are illustrated in Figures 2 and 3.

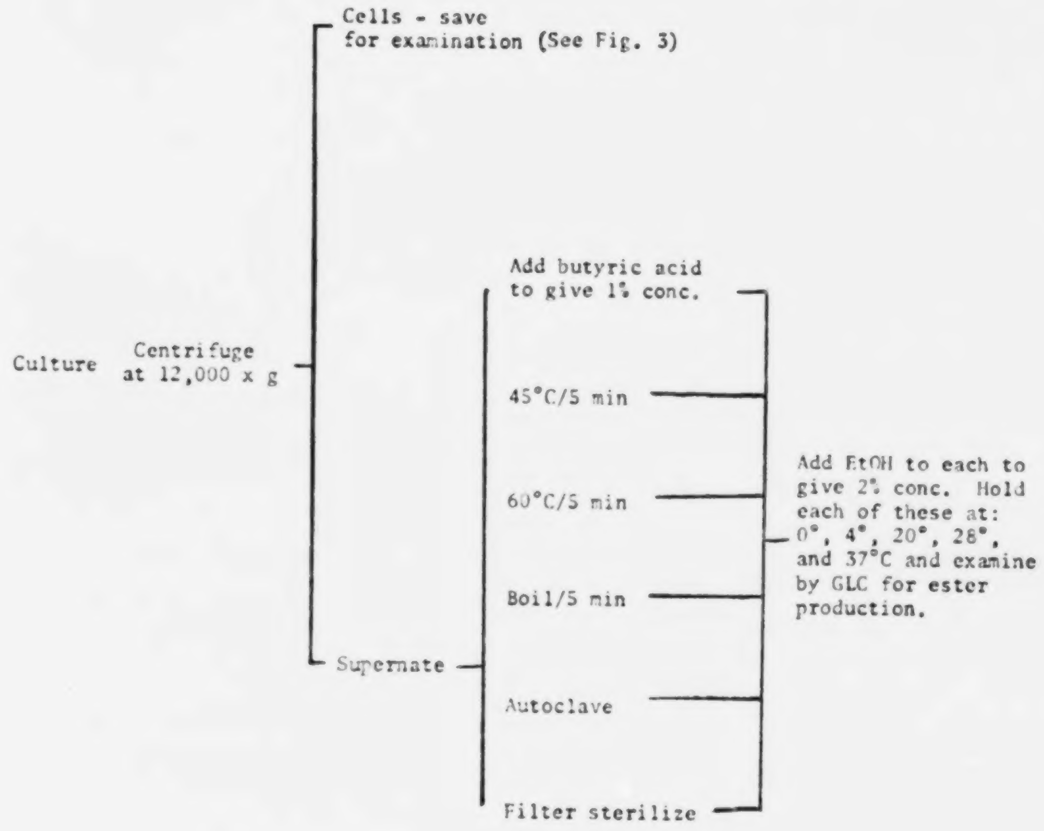

Cells - save
for examination (See Fig. 3)
Culture
Centrifuge
at 12,000 x g
Supernate
Add butyric acid
to give 1% conc.
45°C/5 min
60°C/5 min
Boil/5 min
Autoclave
Filter sterilize
Add EtOH to each to
give 2% conc. Hold
each of these at:
0°, 4°, 20°, 28°,
and 37°C and examine
by GLC for ester
production.

Figure 2. Flow diagram showing procedure used to examine isolate No. 115 culture media for ester production

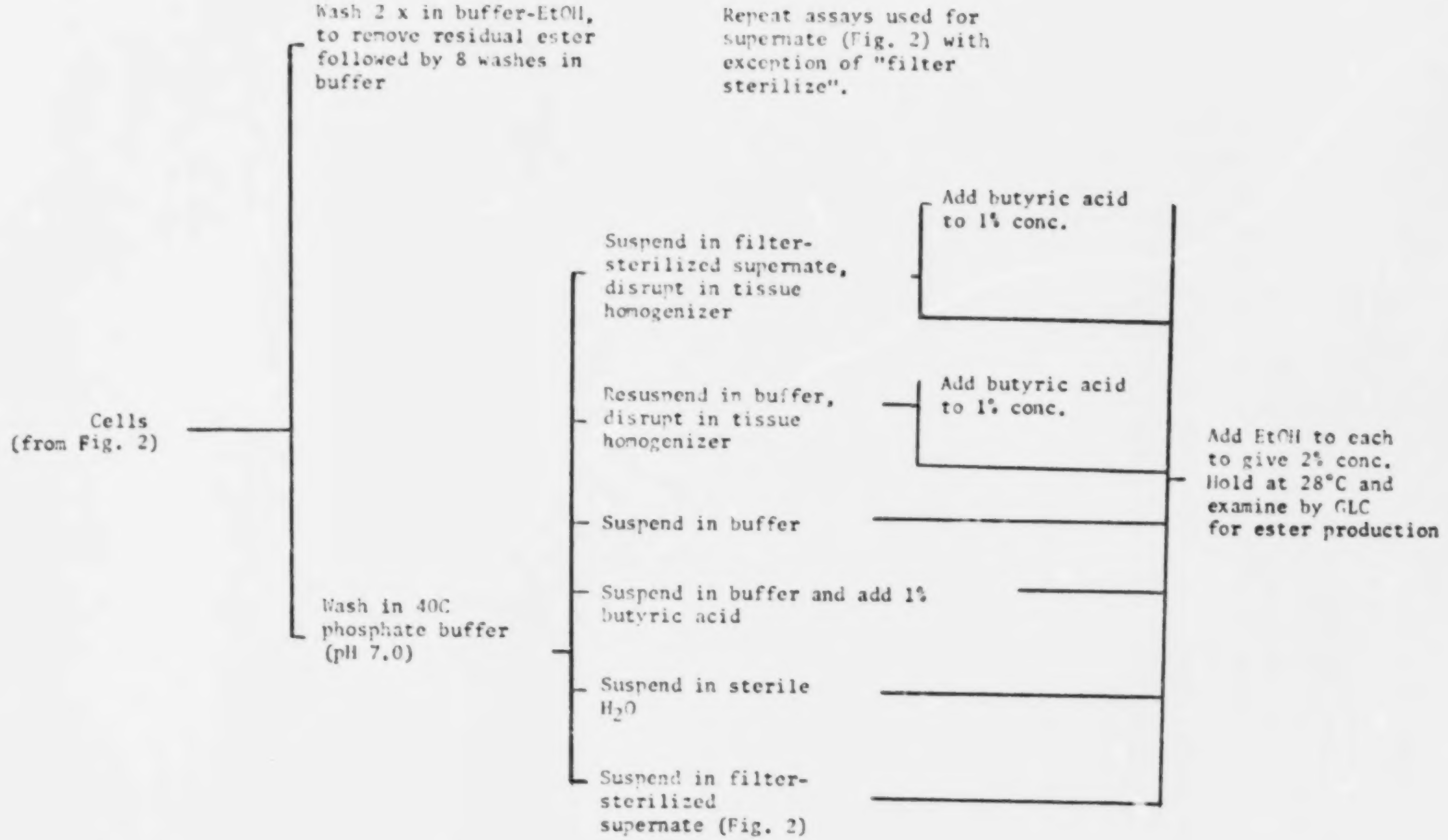

Cells
(from Fig. 2)
Wash 2 x in buffer-EtOH, to remove residual ester followed by 8 washes in buffer
Repeat assays used for supernate (Fig. 2) with exception of "filter sterilize".
Wash in 40C phosphate buffer (pH 7.0)
Suspend in filter-sterilized supernate, disrupt in tissue homogenizer
Add butyric acid to 1% conc.
Resuspend in buffer, disrupt in tissue homogenizer
Add butyric acid to 1% conc.
Suspend in buffer
Suspend in buffer and add 1% butyric acid
Suspend in sterile H_2O
Suspend in filter-sterilized supernate (Fig. 2)
Add EtOH to each to give 2% conc. Hold at 28°C and examine by GLC for ester production

Figure 3. Flow diagram showing procedure used to examine isolate No. 115 cells for ester production

Chromatography of polymer hydrolysate. Purified polymer was added to 2N HCl (1 mg/ml), placed in a glass tube, and sealed. The tube was held at 100°C for 18 hours. The contents were removed and evaporated to dryness at 90°C under a stream of air. Two ml of water were added to the residue and again evaporated to dryness to remove HCl. The evaporation procedure was repeated three times. The residue was then suspended in 0.3 ml distilled water and the hydrolysate (0.02 ml) was spotted onto Whatman No. 4 paper for chromatography. An ascending two-dimensional technique was used (phenol-water, 4:1, w/v and isopropyl alcohol water 4:1, v/v). Individual chromatograms were sprayed with the following reagents: aniline-diphenylamine (86), silver nitrate (86), benzidine (53), aniline-acid-oxylate (23), ninhydrin (86).

The hydrolysis procedure was repeated using 2N H_2SO_4 in place of HCl and portions of the hydrolysate were chromatogramed in the same manner.

Gross Adsorption of Metallic Ions

Gross uptake of several colored metal ions by the cells was used as a screening procedure. Colored metal ions were selected because we could obtain a visual indication of metal accumulation by the color of the cell pellet after centrifugation. The following procedure was used: 0.1 ml of a standardized pre-washed cell suspension in 0.3M phosphate buffer was inoculated into a series of 250 ml Erlenmeyer flasks, each containing 50 ml of trypticase soy broth (TSB, BBL). The flasks were incubated at 28°C for 48 hours on a reciprocal shaker (100 oscillations/min). The cells from each flask were centrifuged and washed twice using 10 ml phosphate buffer and then resuspended in 50 ml of sterile distilled water in 250 ml flasks. One ml each of stock solutions of $CoCl_2$, $CuCl_2$, $FeCl_3$, and $NiCl_2$ were added separately to give the final concentrations of metal ion per flask shown in Table 14. The cell-metal ion suspension was shaken at 28°C for 18 hours on a reciprocal shaker and the cells were then separated from the solution by centrifugation. Accumulation of colored ions in the cell paste at the bottom of the centrifuge tubes was readily seen. Metal ion concentration in the supernatant was determined via atomic absorption spectrometry (Perkin-Elmer) and compared to initial concentrations.

Uptake of Zn^{65}

Accumulation of Zn^{65} by actively growing cells was determined in the following manner: 0.1 ml of a standardized pre-grown washed cell suspension was inoculated into 50 ml of TSB in 250 ml Erlenmeyer flasks. ZN^{65} was added to the flasks in one of three ways: (i) Zn^{65} to give a final concentration of 1μC in 50 ml growth medium (Sp. Act. 1.11 mC/mg), (ii) Zn^{65}, 1μC, plus 50 ug carrier Zn as $ZnCl_2$ in 50 ml growth medium, (iii) Zn^{65}, 1μC, plus 500 ug Zn carrier. The cells were then incubated at 28°C for periods up to 14 days on a reciprocal shaker. Flasks containing the culture were removed from the shaker at specified intervals (see Figs. 30 and 31) and centrifuged. Five ml aliquots of supernatant were removed and counted. The cell paste was resuspended in 3 ml distilled water, mixed by stirring, transferred to a counting vial, and centrifuged again. The supernatant was decanted and considered as a wash. The cell paste was dried in an oven at a maximum of 180°C and then weighed and counted using an automatic gamma well counter (Nuclear Chicago).

Adsorption of Mine Water Ions

Z. ramigera 115 cells were cultivated in 250 ml Erlenmeyer flasks which contained 50 ml of the following basal growth medium: alanine, 0.5 g; arginine, 0.25 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; B_{12} 7.5 x 10^{-8} g; glucose, 10 g; distilled water to 1 liter. After 72-hour incubation at 24°C incubation on a temperature controlled shaker (180 rev/min), the cell flocs were removed by centrifugation and washed three times with sterile distilled water. The washed cell-flocs were added to 50 ml of prepared ion solutions of known concentration. The ion-floc suspension was held on the shaker at 24°C for 6 hours unless otherwise indicated, after which the floc was removed by centrifugation. Flocs were washed two times with distilled water and the wash water was saved for analysis. Adsorption of metal ion was calculated by comparison of supernatant loss, to the concentration in a control flask which contained no cell-floc. Sufficient replicas of each flask were prepared to give triplicate ion analysis and to allow triplicate determination of cell-floc wet weight prior to addition of ion solution.

Cations were determined by atomic adsorption spectrometry (Perkin-Elmer) and anions by standard wet chemical methods.

Adsorption of the Chlorinated Hydrocarbon: Aldrin

Our experimental procedure was as follows: the test organisms were grown in shake culture at ambient temperature (22° ± 2°C) in nutrient broth (8 gm/l, Difco), harvested by centrifugation, washed twice and resuspended in 25 ml of distilled water. Erlenmeyer flasks containing 25 ml suspensions of bacterial floc were then placed on a rotary shaker and 1 ml of aldrin dissolved in acetone was added to give a final concentration of 1 x 10^{-6} g aldrin/26 ml. After being shaken at 120 rpm for the time periods indicated in Figures 37 and 38, the flasks were removed from the shaker and the floc was separated from the supernatant by centrifugation. The flocs were washed twice with distilled water and the washings were added to the original supernatant. The pesticide exposure time was calculated as that period between addition of aldrin to the solution and the separation of the second washing from the bacterial floc. The floc and supernatant fractions were extracted separately with a 3:1 mixture of heptane and acetone. The organic solvent fractions containing the aldrin were concentrated by evaporation at 80°C in glass 15 mm I.D. x 100 mm tubes and adjusted to a volume of 4 ml. Two µl of sample were injected into an Aerograph model 200 gas chromatograph (GLC) utilizing an electron capture detector, 250 mc of titanium tri-tritide, column temperature 185°C, detector 200°C, injector port 225°C, 5 ft glass 1/8 inch internal diameter column packed with Chromosorb W 60/80 mesh, coated with 5% Dow Silicone

SE-30, high purity N_2 gas carrier, 60 ml/min flow rate. The lower limit of aldrin detection by this procedure was about 2.5×10^{-12} g aldrin as determined by a 1.0 cm peak height on the strip recorder.

Samples of natural sediment which were in the process of settling and accumulating in Lake Erie were collected by specially designed sediment collectors placed on reefs (49). This sediment consisted primarily of inorganic clay particles and averaged about 10% organic matter. In addition to being analyzed in a manner identical to that described for bacterial floc, an extract was also examined on a Dohrmann Instrument Co. model 200A microcoulometer, inlet temperature 300°C, furnace temperature 800°C, equipped with a 6 ft glass column, 5 mm internal diameter, packed with Chromasorb W 60/80 mesh, coated with 7.55% of QF-1 and 5.20% D.C. 200, High purity N_2 gas, 190 ml/min flow rate.

Culture and Media for Production of Zoogloea 115 Extracellular Polymer

Zoogloea ramigera 115 as reported by Friedman and Dugan (40) and Joyce and Dugan (56) was employed in this study. The organism was cultured and maintained in the following modification of the arginine medium of Crabtree et al. (27): arginine·HCl, 0.5 g; alanine, 1.0 g; $MgSO_4 \cdot 7HOH$, 0.2 g; K_2HPO_4, 2.0 g; KH_2PO_4, 1.0 g; carbohydrate, 5.0, 10.0, or 20.0 g; vitamin B_{12}, 1.5×10^{-6} g; distilled water to 1 liter. Fructose, galactose, glucose, lactose, mannose, soluble starch, and sucrose were examined individually as the carbohydrate source in the basal medium. The amount of carbohydrate was varied from 0.5% to 2.0% depending on the experiment.

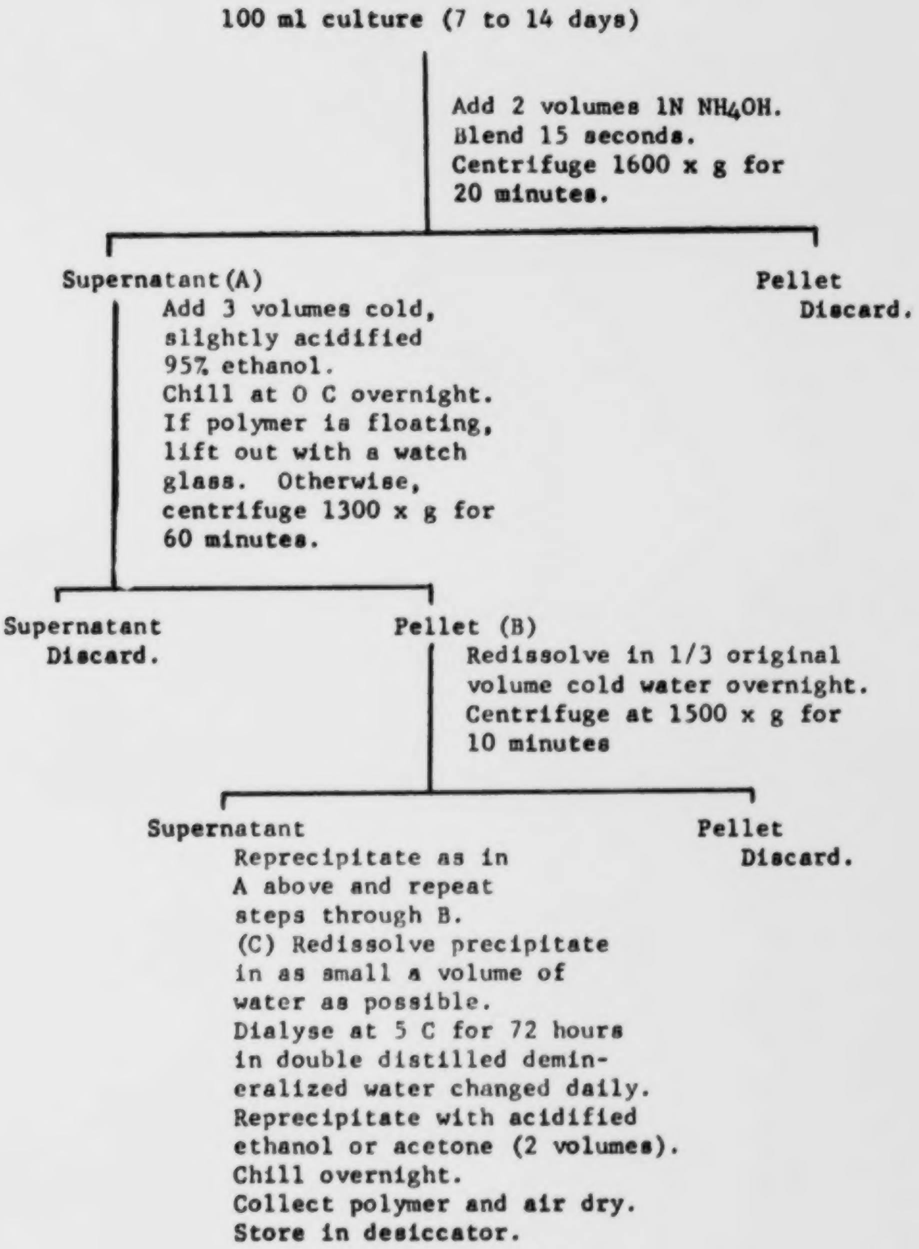
For each carbohydrate source, polymer was isolated from 100 ml of culture incubated in 2.0% carbohydrate in basal medium after 7 to 14 days at 24° ± 2°C on reciprocal shaker (120 strokes/min). To study changes in batch culture and growth medium parameters, a 14 L. fermentor apparatus (New Brunswick Scientific, Co. Inc., New Brunswick, N. J.) was inoculated with a 1% volume of a 72-hour culture and maintained at 24° ± 2°C with continuous agitation (300 rpm) and continuous aeration (3000 cc air/minute) for periods ranging from 7 to 20 days.

Isolation and Purification of Polymer

Polymer from cells grown on each carbohydrate source was treated separately as outlined in Figure 4.

Acid Hydrolysis of Dried Extracellular Polymer

One mg of purified polymer and 1 ml of 2N HCl were sealed in replicate 5" soft glass tubes and hydrolysed at 100°C for either 8.5 or 18 hours. The hydrolysed material was removed from the sealed tube and transferred to a 50 ml beaker which was held in a water bath at 90°C while volatile material was evaporated under a stream of air. A 1.5 ml aliquot of distilled water was added to the hydrolysed residue and again evaporated.



100 ml culture (7 to 14 days)

Add 2 volumes 1N NH_4OH.
Blend 15 seconds.
Centrifuge 1600 x g for 20 minutes.

Supernatant (A)

Add 3 volumes cold, slightly acidified 95% ethanol.
Chill at 0 C overnight.
If polymer is floating, lift out with a watch glass. Otherwise, centrifuge 1300 x g for 60 minutes.

Pellet
Discard.

Supernatant
Discard.

Pellet (B)

Redissolve in 1/3 original volume cold water overnight.
Centrifuge at 1500 x g for 10 minutes

Supernatant

Reprecipitate as in A above and repeat steps through B.
(C) Redissolve precipitate in as small a volume of water as possible.
Dialyse at 5 C for 72 hours in double distilled demineralized water changed daily.
Reprecipitate with acidified ethanol or acetone (2 volumes).
Chill overnight.
Collect polymer and air dry.
Store in desiccator.

Pellet
Discard.

Figure 4. Flow diagram outlining procedure for extraction and isolation of polymer

This procedure was repeated three times to completely evaporate the HCl, then the residue was redissolved in 0.3 ml distilled water and stored under refrigeration for subsequent paper chromatography.

Paper Chromatography

Descending paper chromatography employing Whatman No. 4 paper strips, 19 by 80 cm, and a cylindrical glass tank equilibrated at 24° ± 2°C was used to identify polymer components in the hydrolysed samples. Known samples (100 µg/ml) and polymer hydrolysates (0.1 ml) were spotted on each sheet. Three solvent systems were selected for use: n-butanol-acetic acid-water (4:1:5 v/v), iso-propanol-water (4:1 v/v), ethyl acetate-pyridine-water (12:4:5 v/v). Location reagents included naphthoresorcinol dip (0.2% in acetone, 1 volume to 1 volume of 9% phosphoric acid in water), silver nitrate spray (0.1M $AgNo_3$ in 5M NH_4OH), benzidine dip (0.1 g in 40 ml glacial acetic acid, 30 g trichloroacetic acid, and 40 ml water, 1 volume to 9 volumes acetone), and ninhydrin spray (1.0 g ninhydrin in 500 ml n-butanol, before use add 0.5 ml collidine).

Fermentor Studies and Viscosity Determination

At each sampling time approximately 150 ml of whole culture were removed from the fermentor. Two 10 ml aliquots of whole culture material were quick frozen at -70°C for subsequent determination of dry weight, total deoxyribonucleic acid (30), and poly-beta-hydroxybutyric acid (58). Two 10 ml aliquots of supernatant (6600 x g for 10 min., Sorvall SS-1) were frozen at -70°C for subsequent determination of Nelson's reducing sugar (24) and anthrone sugar (88). An 8 ml aliquot of whole culture and an 8 ml aliquot of the supernatant were used for viscosity determination to estimate polymer production. Precalibrated (#50 B801, #200 Y823, #500 392) Cannon-Fenske Routine Viscometer tubes (Cannon Instrument Co.) were held plumb in a water bath at 23° ± 3°C. Duplicate time determinations were recorded for each sample. The average time in seconds for each sample was multiplied by the temperature corrected viscosity constant for each tube to obtain viscosity in centistokes.

SECTION V

EXPERIMENTAL RESULTS AND DISCUSSION

Nutrition, Biochemistry and Morphology

Several floc-forming bacteria were isolated in this laboratory and others were obtained at various times during the five year span of this investigation from investigators at other laboratories. These isolates were examined using standard microbiological techniques for identification and characterization. Because all isolates were not available during the initial phases of this investigation some have been more thoroughly studied than others and in some instances the organisms were not examined in this laboratory with regard to biochemical characteristics.

Table 1 lists the floc-forming isolates examined during the course of this study and Tables 2, 3 and 4 list results of biochemical reactions for specified floc-formers. During the course of this investigation attention was focused on two closely related isolates of *Zoogloea ramigera* i.e. isolate 115 and isolate 1. Further biochemical data comparing these isolates are presented in Tables 5, 6, 7 and 8.

Crystal violet decolorization is listed as a differential criterion for classification because it was observed that some of the isolates behaved differently when grown on crystal violet agar. Strain 115 is the only isolate observed to decolorize crystal violet; C-1, C-3 and C-22-4 fail to develop colonies on the medium; and I-16-M, P-8-4 and P-95-5 adsorb the dye to produce violet colonies.

The color indicator bromothymol blue is a very sensitive acid indicator and it should be stressed that values recorded in Tables 2, 3 and 4 as VSA are not significantly acid but merely represent a change from the initial pH (7.2) of the medium toward acidity. The difference between values recorded as + and VSA are therefore probably not highly significant.

Comparison of organisms. All isolates are gram negative, polar flagellated rods which flocculate and contain sudanophilic granules under certain growth conditions. None of the Crabtree isolates (I-16-M, P-8-4, P-95-5, C-22-4) have been shown to produce a zoogleal matrix or capsules. Strain 115 of our isolates has been shown to possess a zoogleal matrix, whereas, C-1 and C-3 have not.

Strain I-16-M has been recommended by Crabtree and McCoy as a neotype *Zoogloea ramigera* and comparison of this strain to P-8-4, P-95-5, and C-22-4 has been reported previously (27, 28). C-3 differs markedly from all other isolates with regard to biochemical reactions and is considered to be a flocculent *Pseudomonas* species.

TABLE 1. Floc-forming isolates, source of isolation and reference to information if available

Isolate	Obtained from	Reference
Zoogloea ramigera I	Dugan	56
Z. ramigera 115	Dugan	40, 42, 43
Z. ramigera I-16-M	Crabtree-McCoy	27, 28
Z. ramigera Z-SC-38	Ganapati	44
Z. filipendula P-8-4	Crabtree-McCoy	26, 27
Pseudomonas denitrificans P-95-5	Crabtree-McCoy	26, 27
P. denitrificans ATCC 13867	ATCC[1]	ATCC
Acetobacter suboxydans ATCC 621	ATCC	ATCC
A. oxydans ATCC 6433	ATCC	ATCC
Unidentified gram negative polarly flagellated		
C-1	Dugan-Friedman	40
C-3	Dugan-Friedman	40
C-22-4	Crabtree-McCoy	26, 27

[1] ATCC, American type culture collection

TABLE 2. Biochemical reactions of floc-forming bacteria[a]

Organism	Litmus milk	Methyl red	Nutrient gelatin	Urease	Indole	Citrate	NO_3 reduced	$NO_3 \downarrow N_2$	Casein hydrolysis	Starch hydrolysis	Crystal violet decolorized	Catalase
Zoogloea ramigera												
115	Slightly alkaline	-	-	+	-	-	+	-	+	+	+	+
C-1	Alkaline	-	-	-	-	+	+	-	-	-	NG	+
C-3	Acid	+	+	-	+	+	-	-	+	+	NG	+[b]
Z. ramigera												
I-16-M	Slightly alkaline	-	-	-	-	-	+	-	-	-	-	+
Z. filipendula												
P-8-4	Slightly alkaline	-	-	+	-	-	+	-	-	-	-	+
Pseudomonas denitrificans												
P-95-5	Slightly alkaline	-	-	-	-	-	+	+	-	-	-	+
C-22-4	Slightly alkaline	-	-	-	-	-	+	-	-	-	NG	+

[a] All organisms were cytochrome oxidase positive. None produced sulfide. Reactions are indicated as follows: -, negative response; +, positive response; NG, no growth.

[b] Very slight.

TABLE 3. Reaction of floc-forming bacteria in sugar broth with bromothymol blue indicator

Organism	Arabinose	Cellobiose	Fructose	Galactose	Glucose	Glycogen	Inulin	Lactose	Levulose	Maltose	Mannose	Melezitose	Melibiose	Raffinose	Rhamnose	Salicin	Sorbose	Sucrose	Xylose
Zoogloea ramigera																			
115	VSA[a]	VSA	VSA	SA[b]	•[c]	SA	•	VSA	VSA	SA	VSA	•	•	•	•	•	•	VSA	•
C-1	VSA	VSA	VSA	•	VSA	•	VSA	VSA	•	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA	VSA
C-3[d]	A[e]	A	A	A	A	A	•	A	A	A	A	•	•	•	•	A	•	A	•
Z. ramigera																			
I-16-M	A	•	VSA	VSA	VSA	•	•	•	VSA	SA	•	•	•	•	•	•	•	SA	VSA
Z. filipendula																			
P-8-4	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Pseudomonas denitrificans																			
P-95-5	SA	•	SA	SA	•	•	•	•	SA	SA	VSA	•	•	•	•	•	•	VSA	SA
C-22-4	A	•	A	A	A	•	•	•	SA	VSA	SA	•	•	•	SA	•	•	SA	SA

[a] Very slightly acid, blue green, pH 6.9 to 6.8.

[b] Slight acid, yellow green, pH 6.7 to 6.3.

[c] Growth but no acid, blue, pH 7.2 to 6.9.

[d] Acid was also produced in sufficient quantity to change bromocresol purple-yellow (pH 5.2). All other organisms grew without acid reaction in purple broth base.

[e] Acid, yellow, pH 6.2 to 6.0.

TABLE 4. Reaction of floc-forming bacteria in alcohols and sugar alcohols using bromothymol blue indicator

Organism	Methanol 1%	Ethanol 1%	Propanol 1%	Butanol 1%	Glycerol 1%	Inositol	Mannitol	Adonitol	Sorbitol	Dulcitol
Zoogloea ramigera										
115	+	+	+	±	+	+	+	+	+	+
C-1	+	VSA	VSA	+	VSA	VSA	VSA	VSA	VSA	VSA
C-3	+	+	+	+	A	+	A	+	+	+
Z. ramigera										
I-16-M	+	+	±	±	+	+	VSA	+	+	+
Z. filipendula										
P-8-4	+	VSA	VSA	+	+	+	+	+	+	+
Pseudomonas denitrificans										
P-95-5	+	+	+	+	VSA	SA	VSA	SA	SA	+
C-22-4	+	A	VSA	±	+	+	SA	+	SA	+

+, growth but no acid, blue ±, slight growth but no acid, blue VSA, very slight acid, blue green

SA, slight acid, yellow green A, acid, yellow

TABLE 5. Biochemical characteristics of *Zoogloea* isolate No. 1 and *Zoogloea ramigera* isolate No. 115 when cultured in liquid tubes of purple broth basal medium (Difco) or semi-solid media at 25°C

Test	Isolate No. 1	Isolate No. 115
Litmus milk	Alk[a]	Alk
MR, VP	-[b], -	-, -
Gelatine hydrolysis	+[b] in 72 hr	+ in 72 hr
Gelatin liquefaction	+ in 4 weeks	+ in 4 weeks
Collagen hydrolysis	-	Weak + after 5 days
Chitin hydrolysis	-	-
Urease	+	+
SIM (sulfide, indole, motility)	-, -, +	-, -, +
Citrate, Koser's broth	Growth in 24 hr	Growth in 24 hr
Citrate, Simmon's slant	Alk, 24 hr	Alk, 24 hr
Nitrate (NO_3 to NO_2)	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Catalase	+	+
Crystal violet	Decolorized	Decolorized

[a] Alk = alkaline (color change purple to deep blue except in the case of Simmon's citrate slant which went from green to deep blue).

[b] (+) = positive reaction or presence, (-) = negative reaction.

TABLE 6. Growth of isolates No. 1 and No. 115 in basal medium II plus amino acids as carbon and nitrogen sources; cultures were incubated at 25°C for 72 hr

Substrate Added	Basal Medium II plus					
Supplement	B_{12}[a]		YE[b]		YE + Glucose[c]	
Isolate No.	1	115	1	115	1	115
Alanine	-[d]	+	-	+	-	+
Arginine	-	+	-	+	-	+
Aspartate	-	+	-	+	-	+
Cystine	-	-	-	+	-	+
Glutamate	-	+	-	+	-	+
Glycine	-	-	-	-	-	-
Histidine	+	+	+	+	+	+
Homoserine	-	-	-	-	-	-
Hydroxyproline	-	+	-	+[e]	-	+
Isoleucine	-	-	-	-	-	-
Leucine	-	+	-	+	-	+
Lysine	-	+	-	+	-	+
Methionine	-	-	-	-	-	-
Phenylalanine	-	+[f]	-	+	-	+
Proline	-	+	-	+	-	+
Serine	-	-	-	-	-	-
Threonine	-	-	-	-	-	-
Tryptophan	-	-	-	-	-	-
Tyrosine	-	+	-	+	-	+
Valine	-	+	-	+	-	+
Guanine	-	+	-	+	-	+
Adenine	-	+	-	+	-	+
Cytosine	-	+	-	+	-	+

[a] B_{12} concentration: 1.5 x 10^{-7} µg/ml.

[b] Yeast Extract concentration: 10 µg/ml.

[c] YE concentration: 10 µg/ml; glucose concentration: 20 mg/ml.

[d] (+) = growth, (-) = no growth.

[e] When this medium was supplemented with ethyl alcohol, formaldehyde was produced.

[f] Very slight growth after 72 hr.

TABLE 7. Growth of isolate No. 115 with sugars and sugar alcohols as sole and supplementary carbon sources; cultures were grown at 25°C for 72 hr

	Basal Medium II plus	
Substrate Added[a]	$(NH_4)_2SO_4$[b]	Arginine[c]
Arabinose	+[d]	+
Cellobiose	+	+
Cellulose	-	-
Fructose	+	+
Galactose	+	+
Glucose	+	+
Lactose	+	+
Maltose	+	+
Mannose	+	+
Melibiose	+	+
Melezitose	+	+
Raffinose	+	+
Rhamnose	+	+
Ribose	+	+
Sorbose	-	+
Sucrose	+	+
Xylose	+	+
Arabitol	+	+
Dulcitol	+	+
Erythritol	+	+
Glycerol	+	+
Inositol	+	+
Mannitol	+	+
Sorbitol	+	+

[a] Sugar and sugar alcohol concentrations: 50.0 mg/ml.

[b] $(NH_4)_2SO_4$ concentration: 0.5 mg/ml.

[c] Arginine concentration: 0.5 mg/ml.

[d] (+) = growth, (-) = lack of growth.

TABLE 8. Growth of isolate No. 115 on short chain alcohols and acids when added to basal media

	Basal Medium II plus			
Substrate Added[a]	$(NH_4)_2SO_4$[b]	Time[c]	Arginine[b]	Time[c]
Methyl alcohol	+[d]	< 24	+	< 24
Ethyl alcohol	+	< 24	+	< 24
Propyl alcohol	+	< 24	+	< 24
Isobutyl alcohol	+	< 24	+	< 24
sec-butyl alcohol	-		-	
n-Amyl alcohol	+	72	+	36
Dodecyl alcohol[e]	-		+	40
Lauryl alcohol[e]	-		-	
Stearyl alcohol	-		-	
Ethylene glycol	+	48	+	48
Diethylene glycol	-		-	
Formic acid	-		+	< 24
Acetate (Na)	+	24	+	24
Propionate (Na)	+	48	+	48
Butyric acid	+	120	+	120
Ethyl butyrate	-		+	240
α-ketobutyric acid	-		+	168
α-ketoglutaric acid	-		+	168
β-hydroxybutyric acid	+	120	+	120
Caproic acid	-		+	3[f]
Capric acid	-		+	3[f]
Lauric acid	-		-	
Stearic acid	-		-	

[a] Alcohols and acids were tested in 0.5%, 1.0%, and 2.0% concentrations and showed no variation in results on any concentration.

[b] $(NH_4)_2SO_4$ concentration: 0.5 mg/ml; arginine concentration: 0.5 mg/ml.

[c] Approximate time in hours of appearance of growth.

[d] (+) = growth, (-) = no growth.

[e] Two different suppliers: dodecyl alcohol from Aldrich Chemical Company, Inc.; lauryl alcohol from Merck and Company.

[f] Time in weeks.

C-1, I-16-M, and 115 all differ in at least two biochemical characteristics. Of these three strains only I-16-M produced a significant acid reaction on any of the carbon sources examined. I-16-M appears to ferment arabinose, galactose and maltose to a lesser extent.

Isolate 115 which forms a typical gelatinous or zoogleal matrix also differed from I-16-M in ability to degrade urea, starch, casein, and crystal violet dye. Colonial morphology of 115 is quite similar after 24 hours incubation to colonies of I-16-M. However, 115 colonies develop a rugose appearance upon further incubation (40-72 hr), whereas the colonial appearance of I-16-M remains the same. The photographs shown in Fig. 5 are typical of the colonial morphology of the two isolates being compared. A depressed center is characteristic after 18-24 hr incubation but becomes wrinkled with a moist appearance beyond 24 hr incubation.

Figure 6 illustrates the capsule which surrounds Z. ramigera 115 cells which results in the formation of zoogloeal slime and "finger-like" projections often described as being characteristic of the organism. Figures 6a and 6b show the typical "finger-like" projections often observed in the slime from a trickling filter or other quiescent natural water that contains organic contaminants. Figure 6c is the same organism under phase contrast microscopy after being negatively stained by the Maneval technique. The cells are grouped in individual packets of two or four cells surrounded by capsules and the individual clumps adhere together forming a "finger-like" projection. The same phenomenon can also be seen in Fig. 6d which is prepared in the same manner as 6c but photographed under brightfield illumination. Figure 6e is similar to 6d and shows greater build-up of "finger-like" projections. A typical zoogloeal mass of capsular slime and embedded cells can be seen in Figs. 6f, 6g and 6h. Granules of poly-beta-hydroxybutyric acid are located within the cells which cause them to appear pleomorphic (Figs. 6e, 6f, 6g and 6h).

Z. ramigera I-16-M is shown in Fig. 7a. No extracellular material is observable around I-16-M when grown on the same medium as 115 (Fig. 6), but intracellular granules previously reported to be PHB are present (27). Morphological characteristics of isolate C-3 are shown in Fig. 7b. No extracellular material has been observed, but large quantities of intracellular material accumulate. Continued synthesis of intracellular granular material causes pleomorphism. Since the granule synthesis is related to nutrient and growth conditions, cell size and shape are not desirable characteristics for identification purposes.

Utilization of defined media. Further comparison among I-16-M, 115 and C-3 were made using the defined media A, B and C listed in the section describing characterization of organisms. All of the defined medium variations examined supported flocculent growth of the three isolates although flocculation was not quantitatively consistent. For example, 115 and I-16-M gave the greatest amount of flocculent growth when the

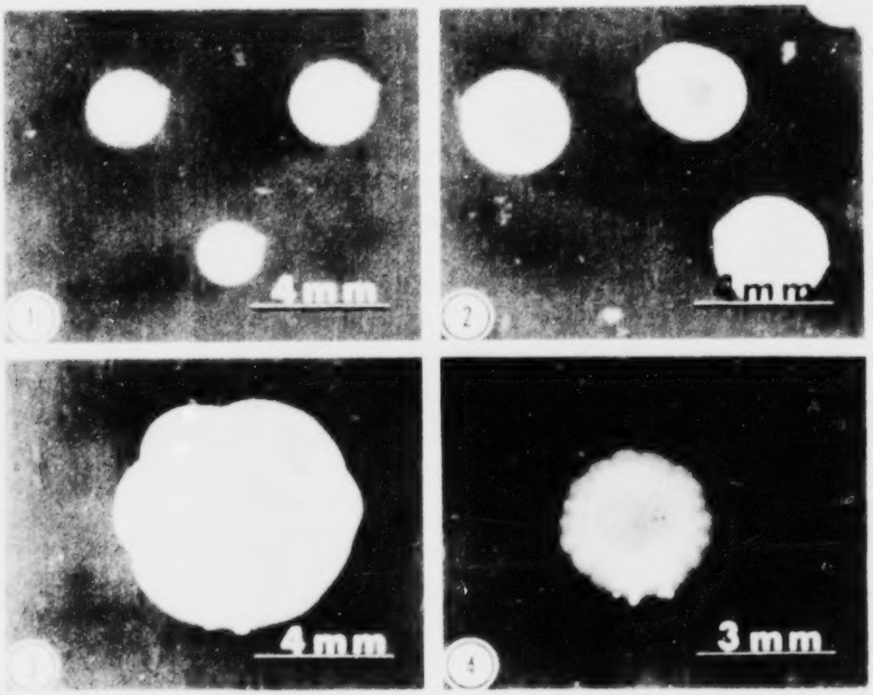


Figure 5. Colonies of *Zoogloea ramigera* 115 and I-16-M after cultivation on tryptone glucose extract agar at 28°C. All colonies are straw colored and appear to be viscous, but are tough and leathery. (1) 115 after 24 hr showing glistening appearance. X5. (2) 115 after 30 hr showing characteristic depressed center of "donut"-shaped colony. X5. (3) 115 after 40 hr showing the rugose appearance. X5. (4) I-16-M after 72 hr showing the depressed center and a crenated periphery. I-16-M does not develop a completely rugose appearance. X7

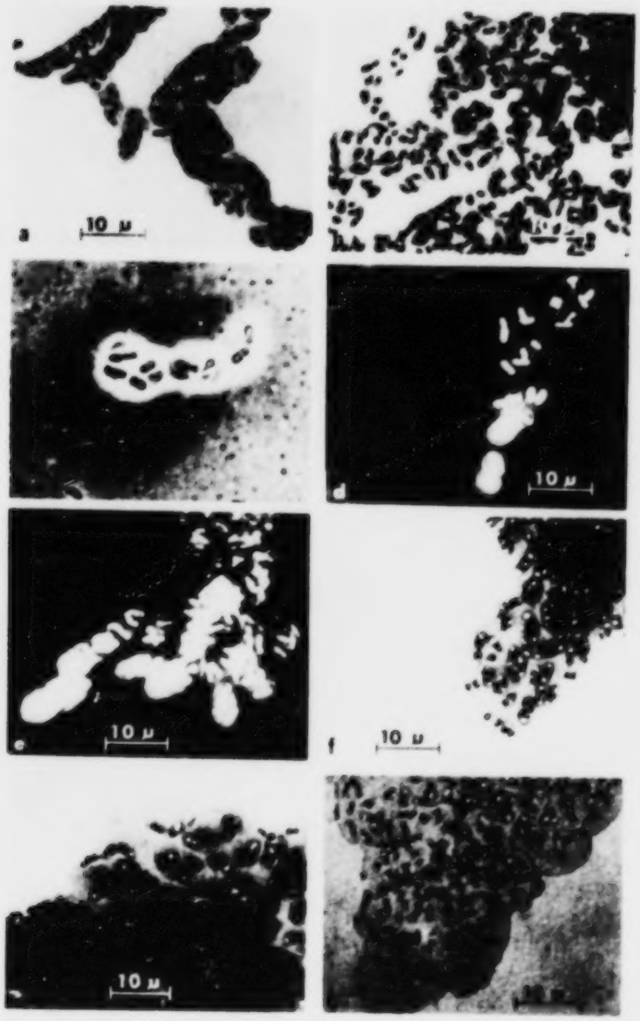


Figure 6. Photomicrographs of isolate No. 115 cells showing "finger-like" projections (a,b); individual cell packets that make up the finger-like projections under phase contrast illumination (c), and under bright field illumination (d,e). 6f, g and h show zoogloeal masses often referred to as the zoogloeal matrix

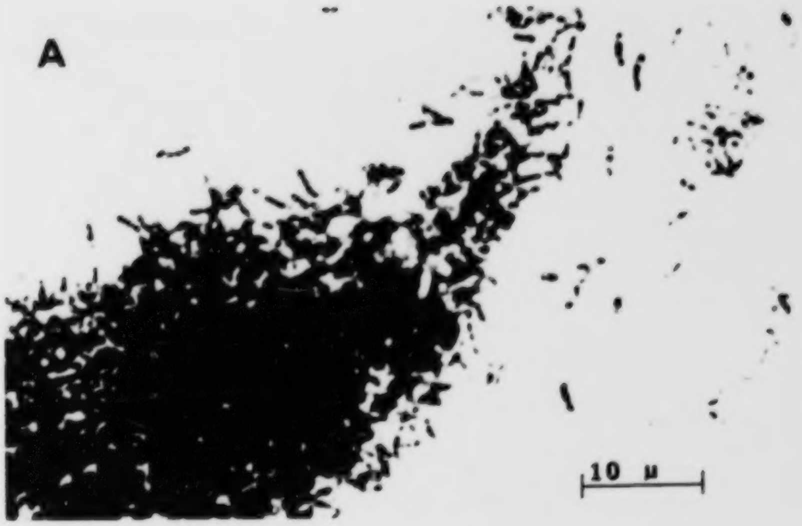
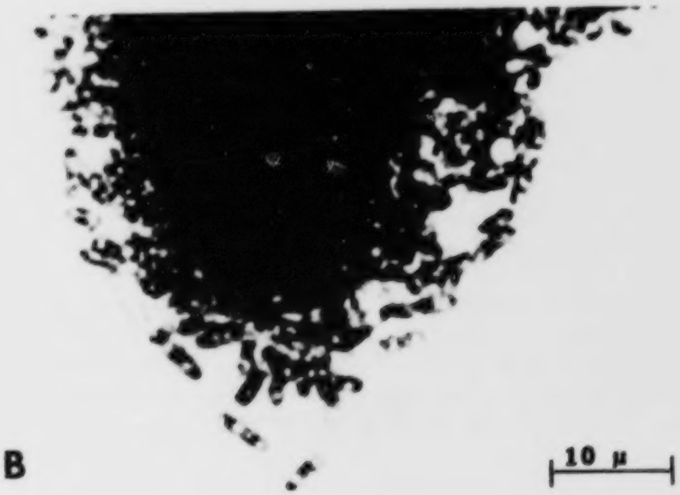


Figure 7. Photomicrographs of a floc of Z. ramigera I-16-M cells (7a) and C-3 cells (7b) showing lack of observable zoogloeal matrix or capsule around cells



media were supplemented with glucose and vitamins. C-3 produced more flocculent growth in the arginine medium (A) when no supplement was added, but it did flocculate in the supplemented medium. The casamino acid medium (C) allowed more rapid growth of the bacteria. The simplest medium which promoted the most flocculent growth was (A) supplemented with glucose and B_{12}. This medium was used in most subsequent biochemical studies. It should also be noted that the cultures grew well in the presence of ammonium ions (medium B) which is contrary to the data of Rich (82).

The generic name *Zoogloea* is derived from Greek roots and literally translates as "living glue". It seems apparent that descriptions in the literature are based upon microscopic observations of the gelatinous matrix or zoogleal material and that this was the unique characteristic of the organisms upon which classification resided. Cultures which do not possess a gelatinous matrix may flocculate but would not necessarily be *Zoogloea* bacteria; that is, floc formation and zoogleal growths are not synonymous. We prefer to consider zoogleal formation as highly polymerized exocellular material analogous to a capsule, and flocculation as a clumping of cells probably resulting from cell surface attractions which probably involve physiochemical influences of capsular material or extracellular polymer fibrils.

Cell size and shape, as well as gelatinous material production, varies considerably with culture media, incubation temperatures, and perhaps oxygen tension in liquid culture media. However, it should be stated that oxygen tension varies with temperature and one influence is not exclusive of the other in our test system. Cell size and shape within limits are, therefore, not adequate criteria for characterizing *Zoogloea* species. Comparison of the Crabtree isolates to our isolates, using identical procedures, shows that a similar pattern of carbohydrate reactions exist among cultures which do produce a gelatinous matrix (115) and those which do not (I-16-M). It is also apparent that cells which flocculate and appear similar morphologically under most growth conditions, do not have similar biochemical reaction patterns (C-3 and 115). That is, C-3 appears to be a *Pseudomonas* or *Aeromonas* species which flocculates. Strain 115 by virtue of the presence of a zoogleal matrix is a *Zoogloea* species according to recognized systems of classification (10). By comparison of 115 to I-16-M, one must conclude that I-16-M is either a nonzoogleal producing *Zoogloea*, as reported by Crabtree and McCoy (29) or a *Pseudomonas* species.

The biochemical reaction patterns of 115 and I-16-M appear to be sufficiently different to conclude that they are different species. However, Unz has isolated 147 strains of floc-forming bacteria based primarily on biochemical differences, all of which he considers to be *Zoogloea* species. (Ph.D. thesis, Rutgers Univ., New Brunswick, New Jersey).

One further point we wish to make regards the observation that both 115 and I-16-M deposit high concentrations of iron around the flocs if iron salts are added to the growth media. Since 115 has a gelatinous matrix around which iron is deposited, one must consider the possibility that it is a species of either the family *Siderocapsaceae* or *Caulobacteraceae*. A discussion of the relationships among iron depositing bacteria and the gelatinous matrix of zoogloea producers is therefore warranted.

DeToni and Trevisan (29) used a gelatinous matrix as a criterion for differentiating sub-families of Coccaceae. A subsequent classification of the same groups of bacteria by Winslow and Rogers (100) used the phrase: "cells aggregated in groups, packets or zoogloea masses" for differentiating all known cocci. Buchanan (9) later suggested that the genus *Siderocapsa* might be sufficiently distinct from other cocci and presented a new key which included *Siderocapseae*. In 1929 Pribram (80) reported on bacterial classification and recognized both rod and coccoid forms of *Siderocapsaceae*. He used the term "zoogloea" although he did not recognize the genus *Zoogloea*. Drake (31) has recently presented a discussion of *Siderocapsa treubii* and considers the work of Hardman and Henrici (46) pertaining to occurrence, distribution, and growth of *Siderocapsa* in alkaline iron bearing waters.

With reference to a possible relationship between *Zoogloea* and species in the order *Caulobacteriales*, Henrici and Johnson (48) described the stalked iron depositing bacterium *Nevskia* on the basis of lobose stalks composed of gum forming zoogloea-like colonies. These latter authors published photomicrographs which show a remarkable similarity to those shown of 115 in Fig. 6.

It is evident that zoogloea producing bacteria are common and ubiquitous in aquatic habitats. Classification of *Zoogloea* is presently based upon highly artificial criteria such as presence of a gelatinous matrix, which is dependent upon growth conditions. Classification of *Siderocapsaceae* is also dependent upon artificial criterion such as deposition of iron around the gelatinous matrix in the natural habitat. This is dependent upon iron content of the natural water environment. We would like to suggest the possibility that investigators in the past would consider the same bacterium as a *Siderocapsa* if it was observed in iron bearing water, or as *Zoogloea* if it was isolated from water rich in organics but extremely low in iron content. Isolate 115 will deposit copper, cobalt, nickle, and presumably other ions in the gelatinous matrix (see subsequent section of report). If these cations had been present in sufficient concentrations in the environment we would have considered it in a different manner.

It is possible to interpret Fig. 6 as a collection of generally spherical-shaped clumps of bacteria, each surrounded by a gelatinous matrix similar to the growth pattern now recognized for Siderocapsa species. Adherance of these spherical gelatinous masses would explain the make-up of what has historically been referred to in *Zoogloea* literature as finger-like projections.

Table 5 presents comparative data obtained from the two isolates using standard biochemical tests in addition to hydrolytic reactions on naturally occurring polymers.

Both isolates were capable of using ammonium salts as the sole source of nitrogen when glucose or ethyl alcohol was supplied as a carbon source. The ability to use amino acids varied in the two isolates. As can be seen in Table 6, isolate 1 was nutritionally more demanding than No. 115. It is interesting to note that growth of isolate 115 on hydroxyproline used as the sole carbon and nitrogen source possessed no odor, but produced an aldehyde odor when supplemented with ethyl alcohol. This product was identified as an aldehyde by the Schiff test (21) and as formaldehyde by the chromotropic acid test (22). No acetaldehyde was detected by the iodoform test (21).

Isolate 115 was capable of utilizing many sugars and sugar alcohols as anticipated from the many published descriptions of Zoogloea (27, 40, 93, 94). Table 7 shows the results of tests made to determine the ability of this isolate to use the sugars and sugar alcohols as sole or supplementary carbon sources. Of the compounds examined, only cellulose and sorbose were unable to serve as sole carbon source. Sorbose did not inhibit growth in the presence of arginine. Presence of cellulose prevented growth in presence of arginine under the experimental conditions. We assume this was due to adsorption of low concentrations of nitrogen or cations by cellulose and not direct inhibition.

Table 8 illustrates the ability of isolate 115 to grow on certain short chain alcohols and acids. The organism eventually grew on most of these substrates tested, but the incubation times required before there was visual evidence of growth varied considerably, and probably reflects an induction period for certain substrate utilizations. When lauric and stearic acids were incorporated in media which normally allowed abundant rapid growth (e.g., PPYE), no growth could be detected at the end of a 4-week incubation period. Ethyl alcohol allowed rapid appearance of growth. The presence of short chain alcohols did not significantly alter the time required for growth to appear nor the quantity of growth as compared to growth in media devoid of alcohols. The presence of short chain acids (propionic, butyric, the alpha-keto acids, and the ester ethyl butyrate), however, resulted in a lag period of 2 to 7 days before growth appeared. Caproic and capric acids inhibited growth in the ammonium salts medium but allowed slow growth (3 weeks) in the arginine medium. Lauric and stearic acids appeared to inhibit growth completely in both media.

Figure 8 illustrates the enhancement of growth in ethyl alcohol supplemented medium over that in nonsupplemented medium.

This suggests that ethyl alcohol is contributing to cell-floc mass (Klett Units) in proportion to alcohol concentration and is independent of the other substrates (PPYE), which is suggestive of growth control by substrates other than carbon.

Because of our previous observation of rapid metal adsorption by cell-flocs (41), we decided to examine the adsorption of organic compounds by flocs. The amino acids alanine and arginine were selected for study.

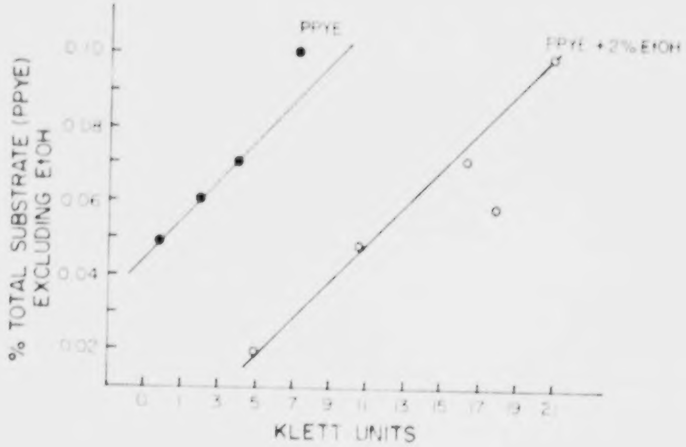


Figure 8. Relative growth (Klett Units) of isolate 115 versus substrate (PPYE) concentration in the presence and absence of 2% ethyl alcohol after 48 hr at 25°C and pH 7.0

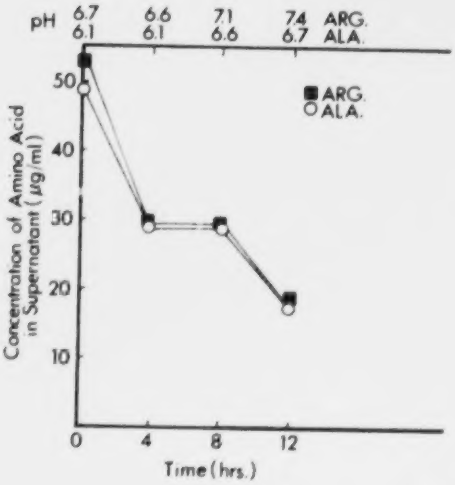


Figure 9. Concentration of arginine or alanine remaining in solution, after removal of cell-floc, versus time. pH of each solution is shown at each time interval

Replicate samples, each containing approximately 20 gm of washed resting cell-floc gel (approx. 0.1 gm dry wt.), were suspended in 50 ml of amino acid solution in Erlenmeyer flasks and held at 24°C on a rotary shaker. Flasks were removed after 4, 8, and 12 hours and the concentration of amino acid was determined in the supernatant after removal of the floc-gel by centrifugation.

Figure 9 shows the concentration of amino acid (quantitative ninhydrin colorimetric reaction) versus time. Approximately 40% of the available amino acid was removed from solution within 4 hours. The concentration remained static between 4 and 8 hours and decreased further between 8-12 hours. The pH of supernatants showed no significant change between 0-4 hours.

We interpret the loss of amino acid between 0-4 hours as adsorption without significant metabolic activity on the amino acid. A rise in pH is observed between 4 and 8 hours, which we interpret as NH_4^+ liberation due to amino acid oxidase activity on the adsorbed amino acid. After 8 hours, sufficient metabolic activity would be present because amino acid degradation would supply nutrient to the cells. Between 8 and 12 hours the pH increases further in correspondence with further decrease in amino acid concentration. We are aware that these projections are not conclusive since data on cell respiration are not yet available.

Zoogloea ramigera isolates 1 and 115 are typical of the floc-producing organisms encountered in aerobic waste treatment processes. Isolate 115 is capable of metabolizing a variety of amino acids as both carbon and nitrogen sources, whereas 1 could not use amino acids, except histidine, as nitrogen sources. Amino acid utilization is accompanied by a pH rise in the medium, presumably due to NH_4^+ liberation. Both isolates were able to hydrolyze casein, gelatin, starch and urea, and isolate 115 could hydrolyze collagen.

A more detailed examination of isolate 115 indicated that the organism could use ammonium as a sole nitrogen source and a variety of organic acids, alcohols, sugars, and sugar alcohols as sole carbon sources. This suggests that the organism probably converts proteinaceous waste material by hydrolysis and amino acid oxidases to corresponding acids or keto acids plus NH_4^+. The acids would be further metabolized using NH_4^+ as the nitrogen source. The organisms are also capable of nitrate reduction, which could produce a suitable nitrogen source in the presence of suitable carbon substrates.

C_{12} and C_{18} alcohols and acids were inhibitory to isolate 115 in the concentrations tested (0.5% = approx. 0.03 molar). Although these concentrations are not likely to be encountered in waste treatment systems, some inhibition might be expected from high fat content waste water.

These, and related bacteria, have been shown to synthesize and store large quantities of poly-beta-hydroxybutric acid (27, 73) and to synthesize extracellular polysaccharide matrix fibrils from a variety of carbon sources (40, 41). This synthetic activity represents a BOD sink or removal mechanism when the flocculent growth settles from suspension.

Isolate 115 has also been shown to produce butyric acid from ethyl alcohol. It is then esterified with residual ethyl alcohol in the growth medium to produce ethyl butyrate. Other esters may be produced by the organisms, depending upon substrate availability, although this has not been studied. Esterified acids would not be recognized as net acid accumulation in growth media or waste water and could be "stripped" from the system by aeration mechanisms. These bacteria in a mixed natural population could be regarded as "buffer" bacteria in that they would neutralize acids produced during high carbon to nitrogen growth conditions (see next section).

The exocellular polymers synthesized by the floc formers have a high capacity for adsorption of metal ions (41) and organic compounds such as amino acids. The adsorption of organic compounds to matrix polymer is analogous to the biosorption described in activated sludge processes by Eckenfelder (see 64) and can explain the exceptionally high rate of BOD removal reported by Butterfield (14, 15) for these organisms.

Ester Synthesis from Alcohol

Growth on alcohol supplemented media. Growth was obtained within 24 hours at 28°C on all media supplemented with C 1 through C 5 alcohols at concentrations below 5%. Higher concentrations appeared inhibitory. Alcohols of length C 6 through C 8 also permitted growth, but the growth was considerably slower, requiring as long as one week to reach the levels obtained with shorter chain alcohols. Lauryl and stearyl alcohol appeared to inhibit growth completely. Short chain alcohols (C 1 through C 5) also could serve as the sole source of carbon in the synthetic medium containing ammonium salts and vitamin B_{12}.

Ester identification. During growth on alcohol supplemented media only, a fruity odor, typical of esters was detected. In the case of ethyl alcohol supplemented media the odor was described as that of fresh pineapple. An infrared spectrum of an absolute alcohol extract of an Aquacide culture supernate concentrate is shown in Fig. 10. Fig. 10B represents the spectrum of an authentic ester (ethyl butyrate, 1% v/v). It can be seen that both spectra show doublet absorption bands at 1720 and 1730 cm^{-1} and a band at 1180 cm^{-1}. These bands are in the regions assigned to the carbonyls of ester groups.

Table 9 lists the retention times of authentic esters and the unknown ester produced in the Z. *ramigera* culture system on an ethylene glycol succinate column at 75°C. It can be seen that ethyl butyrate was the only authentic ester possessing a retention time identical to the culture ester. Similar results were obtained with other columns tested and at lower temperatures.

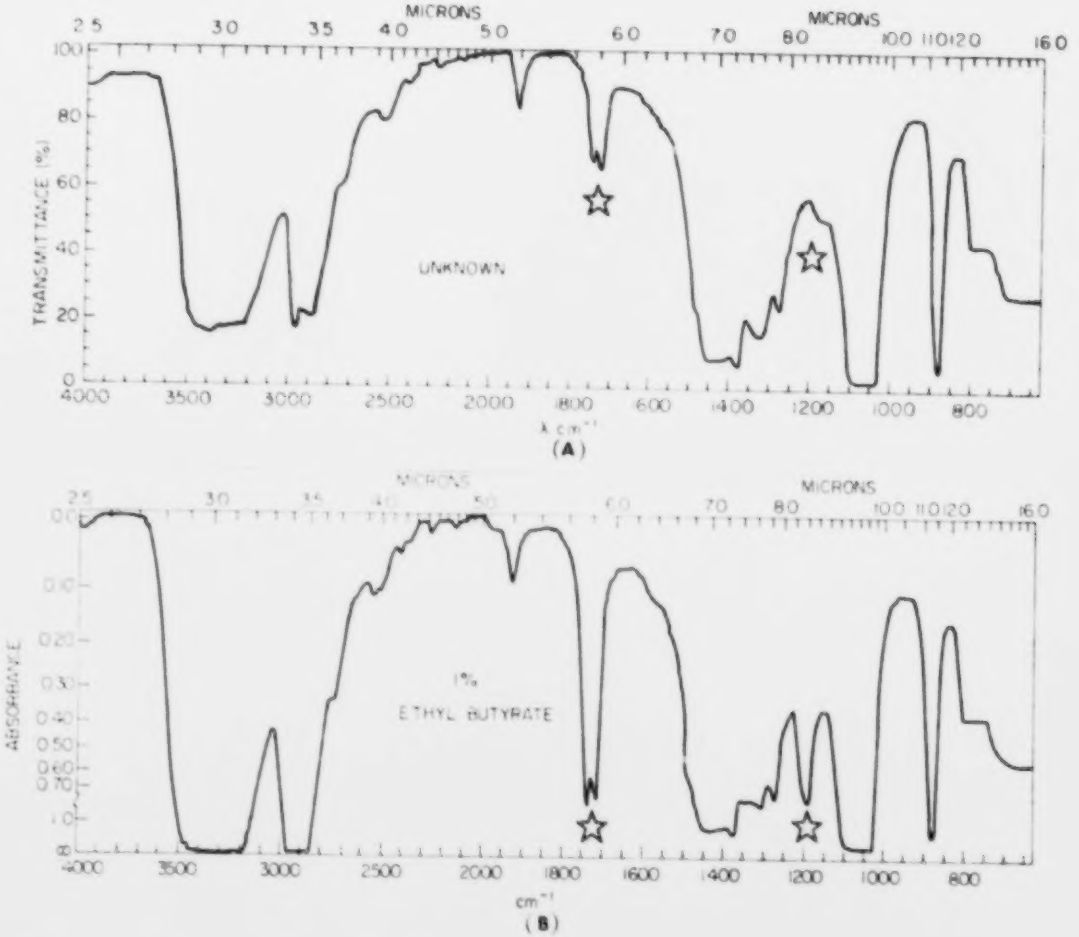


Figure 10. Infrared spectra of A) alcohol extract of Aquacide concentrated culture supernatant; B) control ethyl butyrate. Doublet bands at 1720 and 1730 cm^{-1} and a band at 1180 cm^{-1} (asterisks) are in the region assigned to the carbonyls of ester groups

TABLE 9. Values showing GLC retention times of selected esters on a 15 percent EGS column at 75°C

Ester, 1%	Time (sec)
Methyl propionate	98
Methyl butyrate	98
Ethyl propionate	105
Ethyl butyrate	126
Ethyl propionate	210
Allyl caproate	371
Capryl acetate	315
Culture ester	126

TABLE 10. Values indicating the shortest length of time required for ester detection in culture systems compared to acid catalyzed ester formation in the absence of culture; solutions were held at 25°C.

System plus 2% ethyl alcohol	Time (hr)
Propionic acid + conc. H_2SO_4	24
Butyric acid + conc. H_2SO_4	24
Caproic acid + conc. H_2SO_4	24
Culture (pH 6.8)	4

The response to direct injection of cell-free culture supernates from a PPYE and PPYE-alcohol grown cells into an EGS column at 45°C is shown in Fig. 11. It can be seen that the only response to the supernatant from the non-supplemented media (A) occurred after 360 sec. as a broad peak or series of peaks. The alcohol supplemented culture (B) yielded peaks with maxima at 104 and 149 sec. and a broad response from 483 to 720 sec. The response at 104 sec. corresponds to ethyl alcohol (identical retention time with authentic sample) while the response at 149 sec. represents the culture ester. The responses occurring after 360 sec. in both curves may be due to fatty acids (as suggested by the response to injection of a mixture of unmethylated fatty acids - formic, acetic, propionic, butyric, and lactic - in distilled water) or to as yet unidentified esters present in low concentrations. Figure 12A illustrates the response to injection of an Aquacide concentrate of alcohol supplemented culture supernate. Peaks corresponding to ethyl alcohol and the ester are seen. Addition of authentic ethyl butyrate (0.5% v/v) reinforced the ester peak as seen in Fig. 12B while addition of 2% (v/v) ethyl alcohol reinforced the alcohol peak (Fig. 12C). Figure 13A shows a similar response to injection of an ester extract of alcohol supplemented culture supernate. The alcohol and ester peaks are visible. Figure 13B illustrates the reinforcement of the ester peak when the extract was co-chromatographed with authentic ethyl butyrate (10%).

Nature of ester production. A comparison of the times required for production of ethyl butyrate, in quantities detectable with GLC, by spontaneous esterification of ethyl alcohol and butyric acid in the presence and absence of sulfuric acid and in the presence of non-alcohol and alcohol grown cells of *Z. ramigera* 115 is shown in Table 10. The data indicate the formation of ester in the presence of alcohol grown cells occurs more rapidly than in the absence of cells (spontaneous esterification) or in the presence of non-alcohol grown cells. Reddy (1969) reported the formation of ethyl butyrate by *Pseudomonas fragi* in milk to be quantitatively enhanced by the inclusion of ethyl alcohol and butyric acid in the system. In the case of *Z. ramigera*, inclusion of butyric acid did not appear to speed or quantitatively increase the production of the ester.

The pH of the culture system was in the range of 6.7 to 8.5 when the ester was detected by GLC while the chemical process occurred at pH levels below 6.5. Figure 14 illustrates the pH changes occurring during growth of isolate 115 in media, both alcohol supplemented and non-supplemented. The pH of the non-supplemented and supplemented media dropped from 7.0 to approximately 6.5 during the first four hours. The non-supplemented cultures remained slightly acid for 40 hours when the pH suddenly rose, probably reflecting the formation of ammonium ions due to protein or amino acid degradation. The pH of the alcohol supplemented media rose quickly after the initial drop, reaching a maximum of 8.5 after 48 hours. This pH rise corresponds in time to the detection of the ester in the culture medium. The pH of the alcohol supplemented media dropped to approximately 6.8 sometime after 72 hours. This may reflect

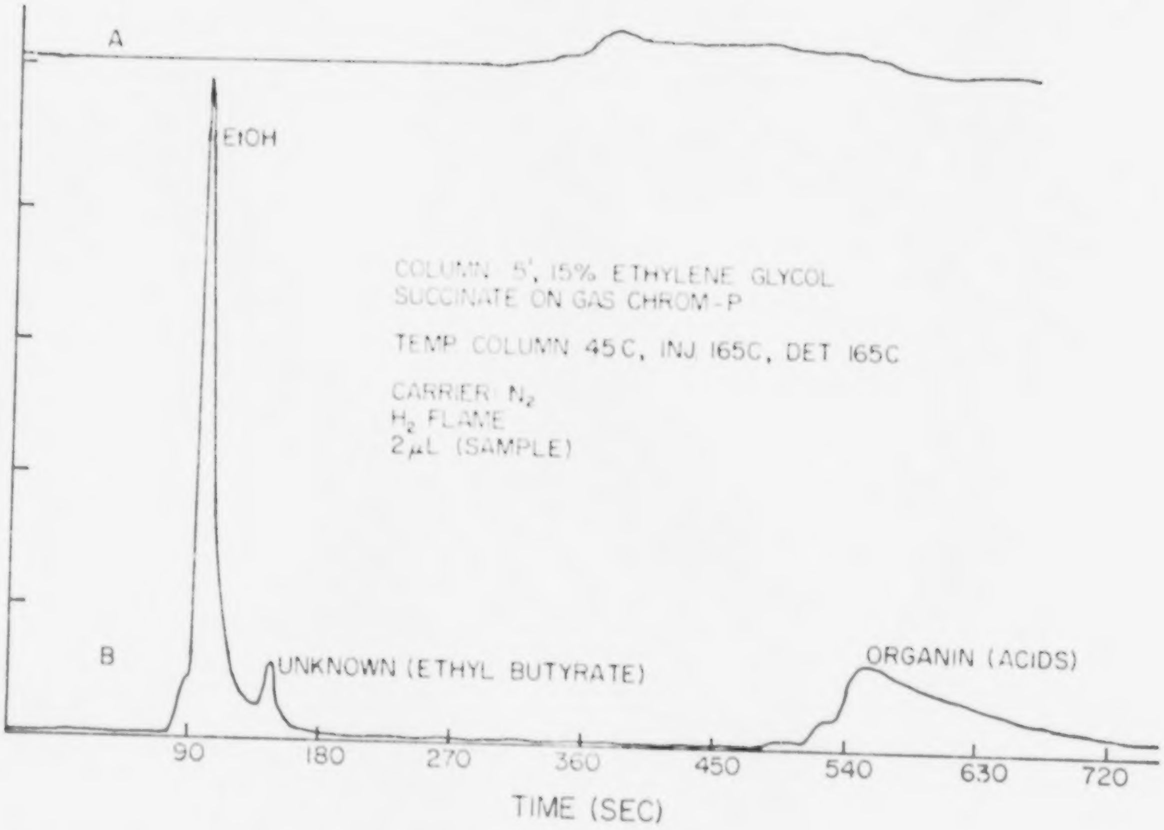


Figure 11. Gas-liquid-chromatograph (GLC) of culture supernatant in presence and absence of ethyl alcohol

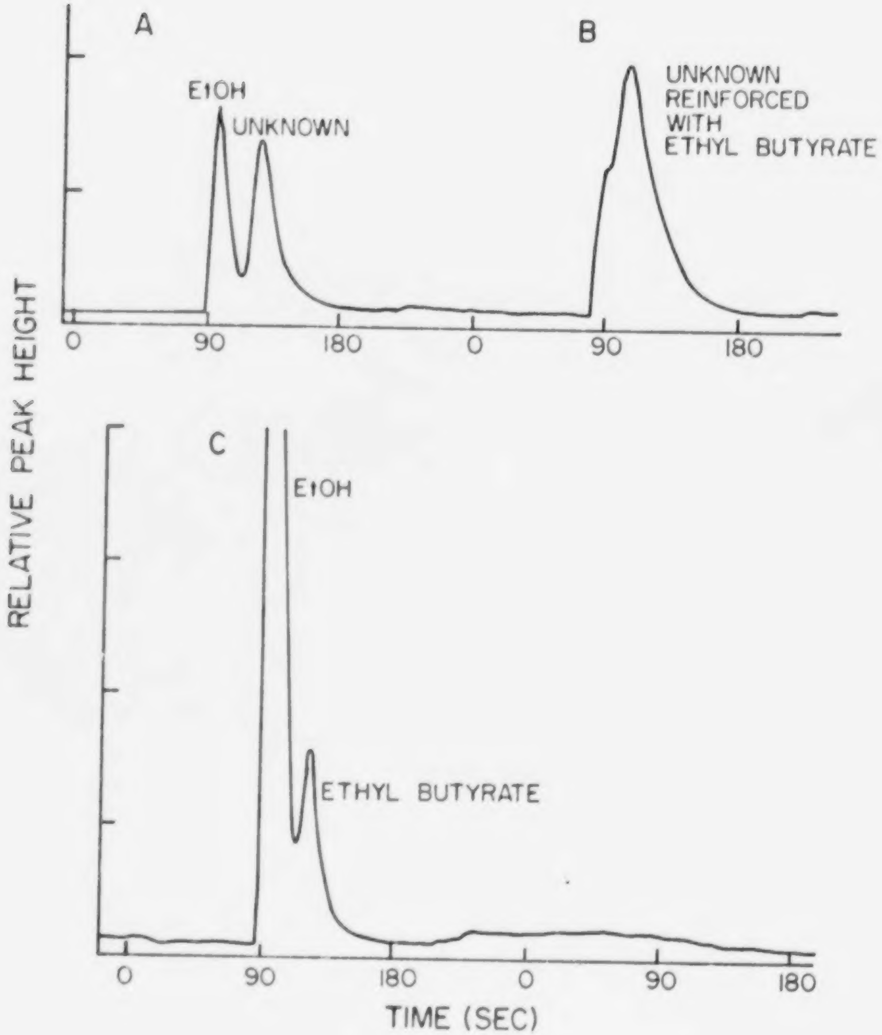


Figure 12. GLC of Aquacide-concentrated culture supernatant in presence and absence of ethyl alcohol

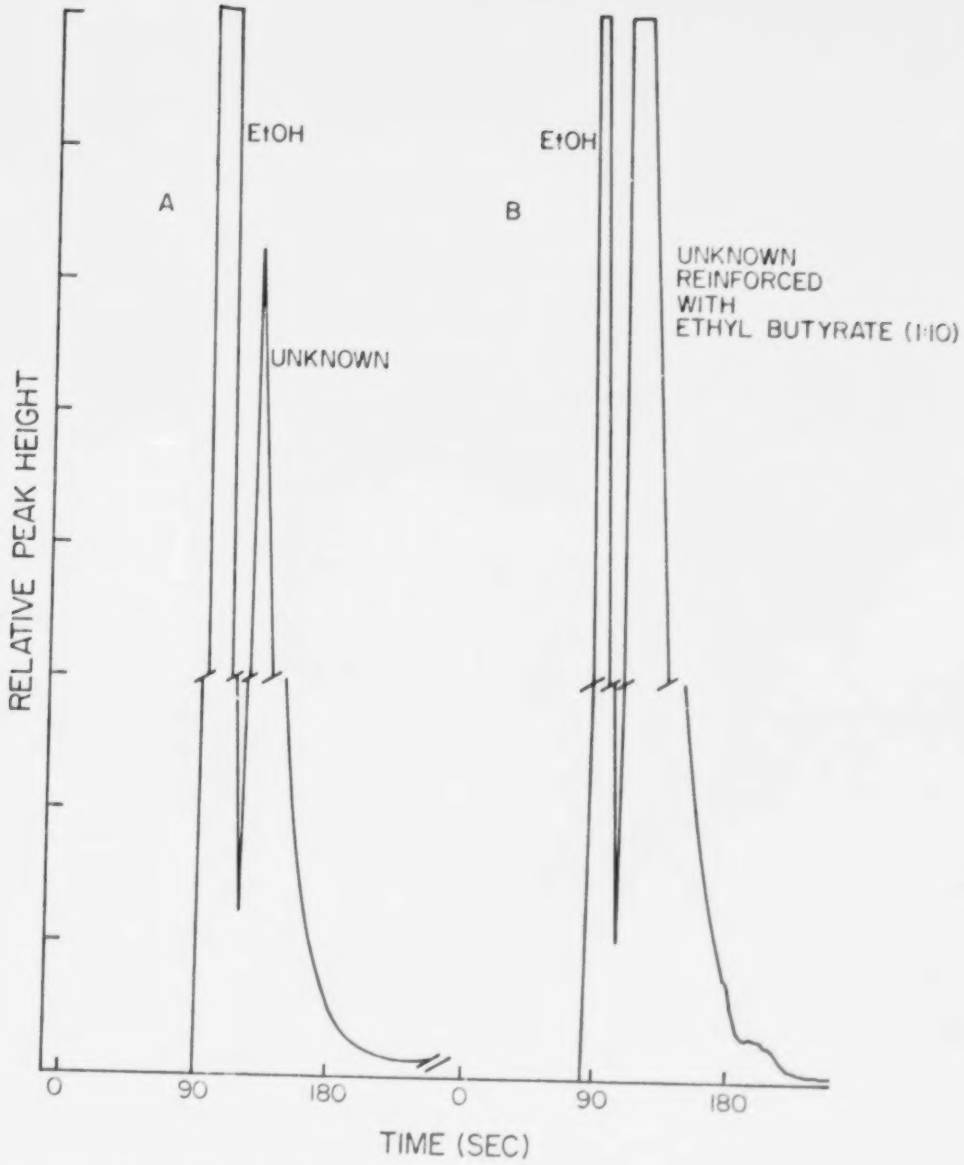


Figure 13. GLC of ether extract of culture supernatant in presence and absence of ethyl butyrate

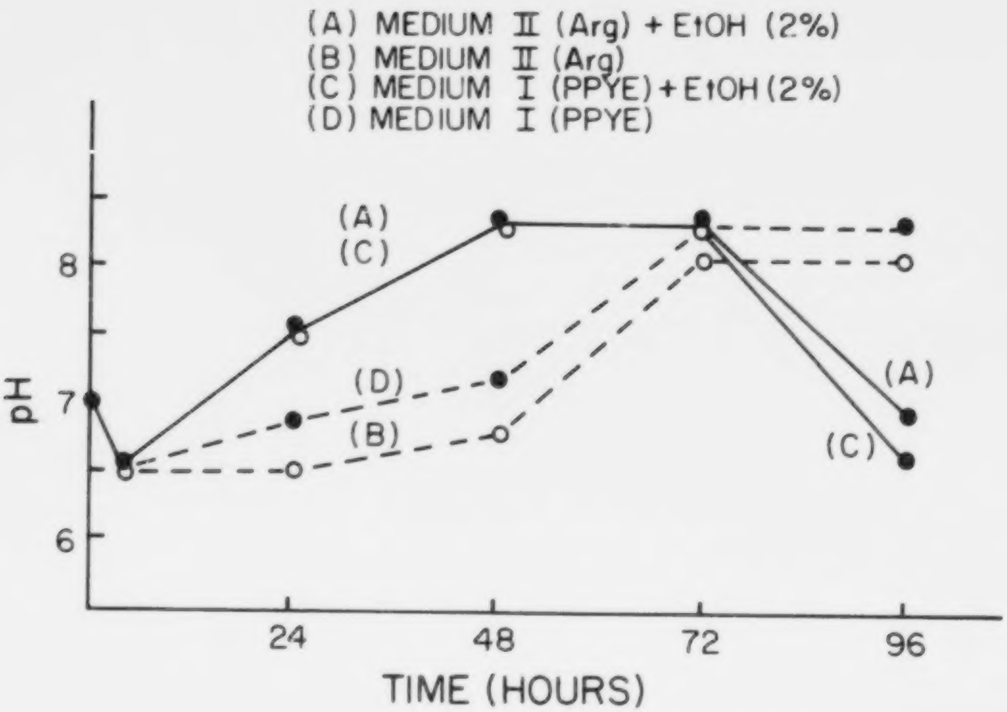


Figure 14. pH changes in ethyl alcohol supplemented and nonsupplemented culture media during growth of *Z. ramigera* 115

the lowering of the alcohol content of the medium and a loss of the already formed ester by volatization. Growth at this point is in the late stationary to early decline phase.

Ethyl butyrate was produced by cultures in ethyl alcohol supplemented media only in the temperature range of 14° to 37°C, with an optimum of 28°C. *Z. ramigera* could grow (multiply) over a range of 4° to 40°C. Transfer of cells grown at 4°C in alcohol media to 28°C resulted in ester production.

Transfer of cells grown in alcohol free media for extended time periods (at least one month) to fresh alcohol-supplemented media did not immediately yield ester formation. However, after several transfers of the cultures in alcohol supplemented media, the ester was again detected.

Examination of cell-free culture supernates for the ability to produce ethyl butyrate suggests the presence of a heat labile factor free in the medium which has a role in ester synthesis. Table 11 illustrates the effects of heat on ester production above the levels formed during culture. Ethyl alcohol was added to the supernate to bring the alcohol concentration to 2% (v/v) as needed. Table 11 shows that the unheated supernate system yielded small increases in ester concentration.

TABLE 11. Ester production in culture supernates which had been heated at various temperatures and resupplemented with 2% ethyl alcohol showing that cultures which had been grown in the presence of ethyl alcohol produced a heat labile substance which was associated with ester production whereas cells which had not been grown in the presence of alcohol produced no ester

Holding Temp. °C	Growth supplemented					Growth not supplemented				
	121°C	100°C	60°C	45°C	U[a]	121°C	100°C	60°C	45°C	U
0	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	+	-	-	-	-	-
28	-	-	-	-	+	-	-	-	-	-
37	-	-	-	-	+	-	-	-	-	-

[a] U = unheated supernate

Addition of unwashed or washed alcohol-grown cells to filter sterilized PPYE culture supernates, heated to 45° or 60°C did not result in the formation of ethyl butyrate. However, addition of these cells to heated PPYE culture filtrate supplemented with 2% ethyl alcohol resulted in the formation of the ester with a maximum yield of 0.5%. Addition of large quantities of these cells to phosphate buffer, pH 7.2, containing 2% alcohol resulted in the formation of the ester in very low concentrations (less than 0.1%); in this case the ester was lost very rapidly, probably due to volatization. Non-alcohol grown cells did not cause ester formation under similar conditions.

The ability of *Zoogloea ramigera* 115 to grow on organic acids and alcohols had been demonstrated (56). Further, the organism is known to produce poly-beta-hydroxybutric acid granules which are observed to be depleted when the organism (27, 73) is placed in a starvation system. This implies that short chain acids, including butyric acid, are being formed.

During the growth of *Z. ramigera* in alcohol free media, no esters were detected, either by gas chromatography or examination of an alcoholic extract of the culture supernate by infrared spectroscopy. Addition of alcohol to the medium, however, resulted in the production of esters by the organism. Ethyl butyrate was identified as the only detectable ester formed during culture in ethyl alcohol.

Formation of the ester is catalyzed by a heat-labile factor(s) found in either the culture medium or cells. This factor esterifies butyric acid produced as a metabolic by-product and residual ethyl alcohol in the medium. The catalytic agent is characteristic of an enzyme in its heat lability, functional temperature, and optimal pH, although no isolation or purification has been made.

We believe this esterification procedure may permit the rapid removal of acids which are formed as metabolic by-products and may explain why the organism has been reported not to have an acid reaction on organic substrates. Organic acids and alcohols present in waste waters would be in low concentration, but the supply would be omnipresent due to the metabolic activities of other microorganisms. Removal of acid products could be of survival value to the organisms. We have observed the formation of ethyl butyrate in a mixed culture system containing *Z. ramigera* 115 and a saccharolytic yeast (unpublished data), suggesting that the ethyl alcohol formed by the yeast was being esterified by the bacterium. Although no data are presented, there is no a priori reason why other alcohols would not also be esterified if present in the growth medium. This would be the case in sewage.

Furthermore, *Z. ramigera* is suspected of being related to *Acetomonas* and *Acetobacter*, organisms associated with C_2 metabolism. We believe this esterifying type of metabolic activity could be responsible for the formation of bouquets in wines and vinegars and also aids in converting undesirable sewage odors to less objectionable odors.

Antigenic Relationships Among the Floc-Forming Bacteria

Immunodiffusion reactions between rabbit antisera and trichloroactic acid soluble (polysaccharide, carbohydrate) antigens are summarized in Table 12. Antisera to *Z. ramigera* 115 reacted with other *Zoogloea* isolates, the C-1 and C-3 organisms and *Acetobacter suboxydans*. No reaction with *Acetobacter oxydans* or the *Pseudomonas* isolates tested could be demonstrated.

Zoogloea I-16-M antisera also reacted with the other *Zoogloea* isolates. However, no reaction with *A. suboxydans* could be demonstrated; whereas it did react with *A. oxydans* and the *Pseudomonas* isolates tested.

Of the antisera tested, none had identical reaction patterns. This is expected because each organism had been considered on the basis of biochemical and morphological examination to be different organisms. The extensive amount of reaction demonstrated among the isolates is indicative

TABLE 12. Summary of immunodiffusion reactions

Antigens (TCA extracts)	Antisera Z. 115	Z. I-16-M	Z. P-8-4	C-3	621 A.sub	6433 A.ox
115 *Z. ramigera*	+	+	+	+	+(2F)	-
I-16-M *Z. ramigera*	+wk	+	+	+wk	+wk	+
P-8-4 *Z. Fillipendula*	+wk	+wk	+	-	+wk	-
C-3 G(-) rod	+	+	+wk	+	-	-
ATCC 621 *A. suboxydans*	+(2F)	-	+wk	-	+	+
ATCC 6433 *A. oxydans*	-	+wk	-	+wk	+	+
C-1 G(-) rod	+	-	-	+		
C-22-4 *Pseudomonas* sp.	-	+	-	-		
ATCC 13867 *Ps. denitrificans*	-	+	+	-		

+ = immunodiffusion reaction observed
- = immunodiffusion reaction not present
2F = 2 fused lines; i.e., lines of identity

of phylogenetic relationship among them. Typical reaction patterns are presented in Fig. 15.

Also of particular significance are the strong double lines of identity between A. suboxydans and Zoogloea 115. These are clearly shown in Figs. 15A and 15B. This suggests that Zoogloea ramigera 115 is closely related to A. suboxydans - perhaps more closely related than A. suboxydans and A. oxydans. However, biochemical and morphological data indicate that Z. 115 and A. suboxydans are not identical.

Reference to Table 12 indicates that antisera to C-1, C-22-4 and ATCC 13867 were not prepared nor were antigens to these organisms tested against A. suboxydans or A. oxydans. Reciprocal reactions were carried out with all other isolates. The only reciprocal reaction which could not reaffirm its counterpart reaction was with C-3 and A. oxydans; i.e. C-3 antisera reacted weakly with A. oxydans antigen but A. oxydans antisera did not react with C-3 antigen.

Deoxyribonucleic acid (DNA) density and mole % G/C

DNA densities were calculated from the position of E. coli reference DNA (1.710 density and 51% G/C) by the following equation:

$$(\text{density})\ \rho = \rho_0 + 4.2w^2 (r^2 - r_0^2) \times 10^{-10}\ g\ cm^3$$

where:

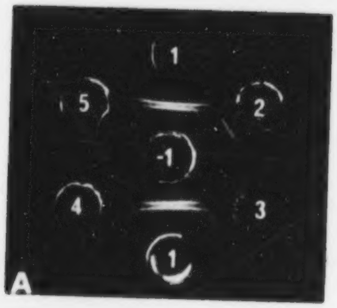
ρ_0 = density of reference DNA
w = speed of ultracentrifuge rotation in radians sec^{-1}
r_0 = distance of reference DNA from center of rotation
r = distance of sample DNA from center of rotation

Mole % of G/C was calculated from the following equation:

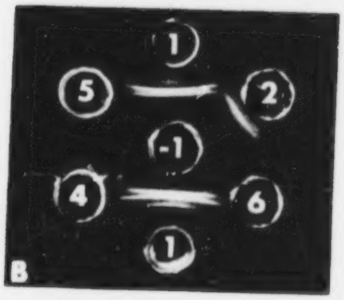
$$\frac{\rho - 1.660}{0.098} \times 100$$

Values are presented in Table 13 and indicate that all of the organisms with the exception of A. oxydans and Z. filipendula P-8-4 had GC ratios in the range of 60 to 64%. This supports the data indicating immunologic relatedness although it is surprising that A. oxydans and Z. filipendula P-8-4 had considerably lower GC ratios.

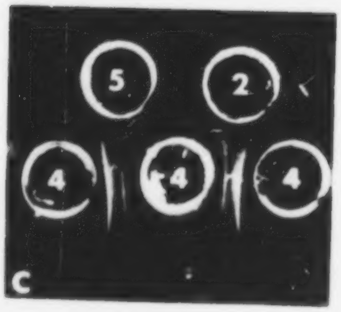
Smooth-rough colony variants of Z. ramigera 115 have been observed on agar plate cultures. Also we have observed vastly different cell morphology with 115 when cultivated in the cold. Consequently DNA analyses were made on smooth, rough and 5°C cultivated organisms in addition to normal shake flask grown cells. Each is listed separately in Table 13. Comparisons of isolates must be made in conjunction of other types of data previously reported (e.g. biochemical, immunological).



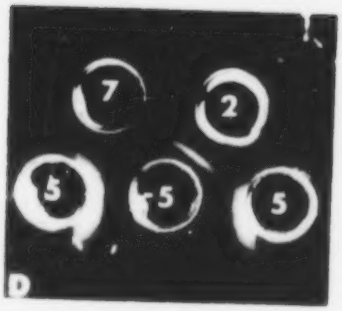
1. A. suboxydans
2. Z. ramigera 115
3. A. oxydans
4. Z. filipendula P-8-4
5. Z. ramigera I-16-M
-1. A. suboxydans antiserum



1. A. suboxydans
2. Z. ramigera 115
6. C-3
4. Z. filipendula P-8-4
5. Z. ramigera I-16-M
-1. A. suboxydans antiserum



2. Z. ramigera 115
4. Z. filipendula P-8-4
5. Z. ramigera I-16-M
-4. Z. filipendula P-8-4 antiserum



2. Z. ramigera 115
5. Z. ramigera I-16-M
7. C-22-4
-5. Z. ramigera I-16-M antiserum

Figure 15. Photographs of typical immunodiffusion reactions between rabbit antisera (center well) and trichloroacetic acid (TCA) antigens from floc-formers

TABLE 13. Summary of deoxyribonucleic acid (DNA) density and base ratio (G/C) analysis

Isolate	Density relative to *E. coli* DNA	mole % Guanine + cytosine (G/C ratio)
Zoogloea ramigera 115	1.7224	63.7
Zoogloea ramigera 115 (rough)	1.7199	61.1
Zoogloea ramigera 115 (smooth)	1.7226	63.9
Zoogloea ramigera 115 (5°C)	1.7225	63.8
Zoogloea ramigera I-16-M	1.7232	64.5
Zoogloea filipendula P-8-4	*	51*
Pseudomonas denitrificans P-95-5	1.7222	63.5
Pseudomonas denitrificans ATCC 13867	1.7235	64.8
Acetobacter suboxydans ATCC 621	1.7188	60.1
Acetobacter oxydans ATCC 6433	*	51*
C-1	1.7206	61.8
C-3	1.7195	60.7
C-22-4	1.7211	62.3
Escherichia coli standard	1.710	51

* could not be differentiated from E. coli DNA standard, 1.710 = 51% GC

All floc-forming isolates examined via immunodiffusion analysis have not been analyzed by nucleic acid density (% G/C). This is because the two studies were carried out at different times and each procedure is fraught with different types of experimental difficulty. For example, the antibody stimulating capacity of different isolates varies considerably and in some cases required that the immunization of animals be repeated with considerable delay in time. We ultimately used Freunds complete adjuvant to stimulate antigenic response in the rabbits. Also, in the case of nucleic acid analysis the procedure of Marmur had to be modified with some isolates because the copious amount of polysaccharide produced by the cells either adsorbed salts from the reagent or adsorbed DNA.

Complete profiles on all of the isolates must await further experimentation.

It is perhaps worth mentioning that we have conducted a computer taxonomic analysis and have compared 22 isolates of *Zoogloea* and floc-forming pseudomonads to 318 bacteria isolated from Lake Erie over a three year period. The interesting result of this analysis is that all 22 isolates clustered at a 90-100% similarity level and suggests a strong relationship among all of the floc-forming isolates examined.

The combined immunologic, GC ratio and computer data are strongly suggestive of a close phylogenetic relationship among the floc-forming bacteria classified as *Zoogloea*, *Acetobacter* and *Pseudomonas* and that many bacteria within this related group appear to be more closely related to each other than they are to other species within the genera in which they are currently placed.

Structure and Composition of Polymer Surrounding Z. ramigera 115

As mentioned previously the zoogloeal matrix which has historically been described as a finger-like projection (10, 14, 33) can be seen in the photomicrographs of isolate 115 shown in Figs. 16-1, 16-2, and 16-3. Figure 16-4 is a phase-contrast photomicrograph of individual packets of isolate 115 cells. We have previously reported that the finger-like projections appear to consist of individual packets of cells (40). The arrows in Fig. 16-3 point to interfaces between individual cell packets which have been stained using dilute crystal violet.

Examination of the zoogloeal flocs in the native state using the freeze-etching technique and subsequent electron microscopy revealed the floc structure shown in Fig. 17. Matrix structure resembling or analogous to the individual packet structure shown in Fig. 16-4 can be observed; an interface (I) between two floc packets can also be seen. Cells embedded within the matrix are not readily observed in Fig. 17-1; however, Fig. 17-2 clearly shows the presence of a cell (C) embedded within the matrix. The matrix appears to consist of polymer which forms a network of fibrous strands (S) composed of two units of different diameter, one measuring 40-60nm and the other 4-5nm. Figure 18-1 illustrates exocellular strands adhering to cells of isolate 115.

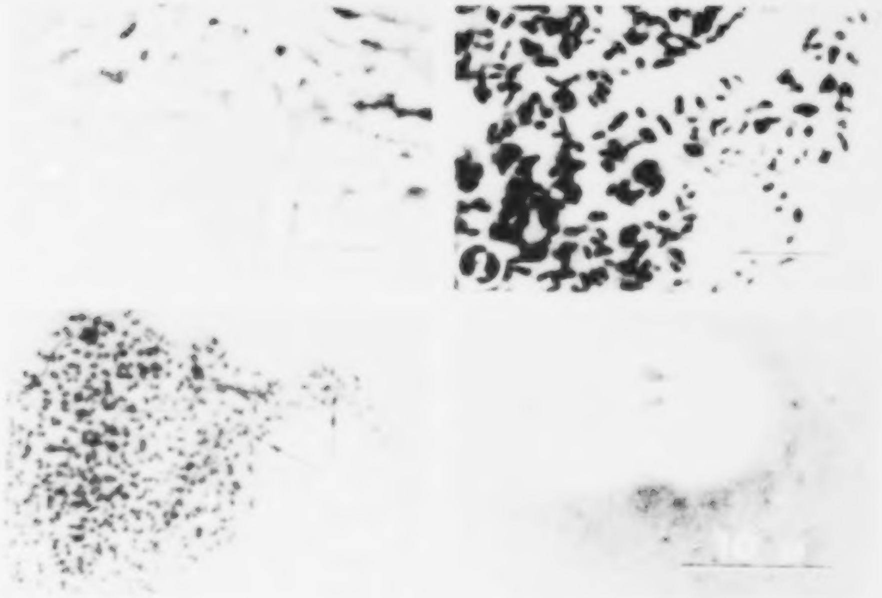


Figure 16 (1). Z. ramigera 115 fingerlike projections composed of cells embedded within the zoogloeal matrix. Cells were suspended in 42% bovine serum albumin

(2). Z. ramigera 115 flocs stained with 1.0% crystal violet

(3). Z. ramigera 115 flocs stained with crystal violet, showing interfaces (I) between individual clumps of cells

(4). Phase-contrast photomicrograph of Z. ramigera 115 cells stained by Maneval's method and showing individual packets of cells. Each packet is surrounded by capsular or zoogloea matrix

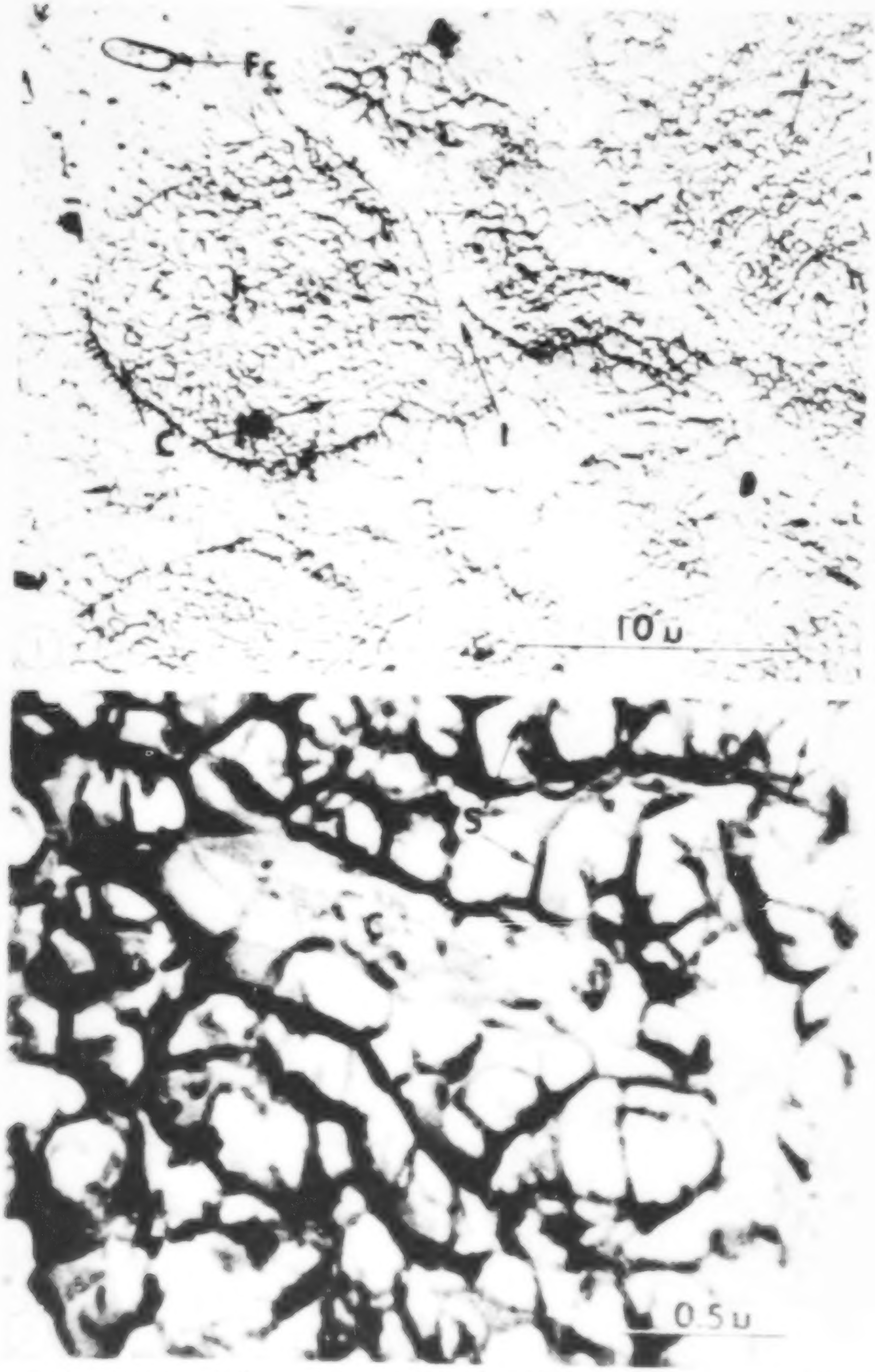


Figure 17. Matrix of *Zoogloea ramigera* 115. (1) Freeze-etching of 115 showing cell-flocs (F). Cells (C) within the flocs are present. Interfaces (I) are present between the cell-flocs. X4,000 (2) Freeze-etching of single cell (C) embedded within zoogloeal matrix. Strands (S) are present around the cells; the thicker strands are 400-600 A, while the thinner strands are 40-50 A. X50,000

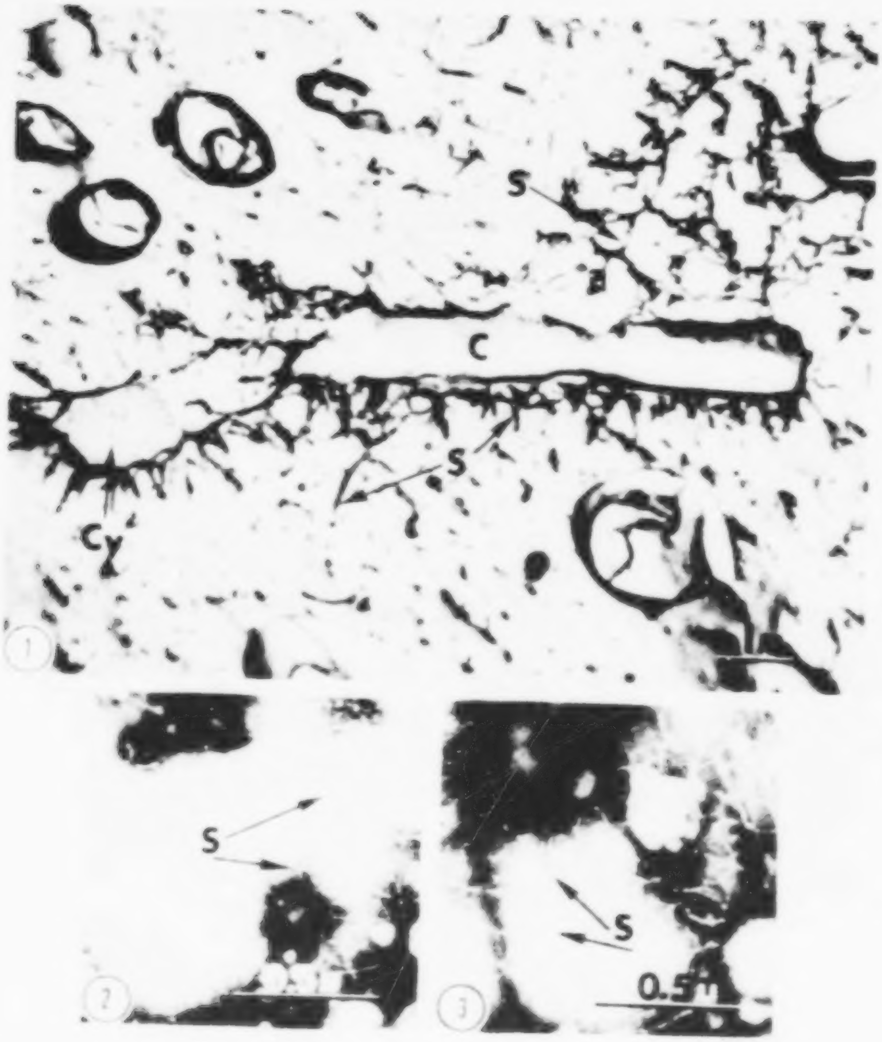


Figure 18. Comparison of strands of Zoogloea ramigera 115 by freeze-etching and negative staining. (1) 115 surrounded by strands (S) of exocellular matrix. The cytoplasm (Cy) may be seen; several free cells are also present. X11,300 (2) Negative stain of matrix surrounding 115 cells. X42,000 (3) Another negative stain of matrix. Strands (S) are present and measure 60-130 A X42,000

The floc matrix has also been examined after negative staining. This technique (Fig. 18-2, 18-3) also reveals strands resembling those observed in frozen-etched specimens. The strands in this micrograph are 6-13nm in diameter.

Figures 19-1 and 19-2 are thin sections of zoogloeal flocs which have been embedded and stained with lead citrate. The extracellular strands can be detected as branching fibrils with a diameter of 4 - 6 nm. Figure 19-2 shows void space (V) surrounding cells embedded in the matrix. This could result from motility within the floc, from lack of polymer synthesis at this location on the cell surface, or it may consist of substances undetectable using this technique. A characteristic gram negative cell wall is present and the cell appears to be packed with ribosomes.

Purified extracellular matrix material has also been examined with the electron microscope and is shown in Fig. 20. Polymer strands resembling those shown in the native state (Figs. 17, 18, 19) can be seen and measure approximately 2 - 5 nm in diameter. An infrared absorption spectrum of the purified material shown in Fig. 20 is presented in Fig. 21. The following functional group assignments were made: OH, 2.95 µm; C-H, 3.38 - 3.44 µm; C=0 of ionized carboxyl or aldehyde, 6.15 um and 7.1 µm; weak C=0 of aldehyde, 5.8 µm and 7.3 µm; tertiary CH-OH, 8.65 µm; band typical of cellulose or polysaccharide ring, 9.4 µm. The I-R spectrum is identified as that of a polysaccharide which may contain carboxyl functions or terminal aldehyde groups.

Chromatograms of polymer hydrolysate (HCl or H_2SO_4) revealed a single spot when sprayed with either aniline-diphenylamine or aniline-acid-oxalate. These reagents developed spots which had an Rf value identical to glucose in the solvent systems employed. The spot color was also identical to that of glucose. It was therefore concluded that glucose is the primary and possibly the only constituent of the polymer. However, subsequent examinations revealed the presence of galactose in the polymer. Several ninhydrin positive spots were present in crude polymer extracts but disappeared during the polymer purification procedure and this was considered evidence of polymer purity. Although the I-R spectrum was interpreted as possibly containing carboxyl groups, no uronic or other organic acids could be demonstrated.

The polymer was not susceptible to hydrolysis by either alpha or beta amylase under the described conditions. The polymer and whole 115 cell-flocs repeatedly gave significant positive responses to cellulase in comparison to carboxymethylcellulose controls.

The only other available Z. ramigera (isolate I-16-M from Crabtree and McCoy) (26, 27) culture has been examined for comparative purposes. Figures 22-1 and 22-2 are shadow-case preparations of cells which were treated with 1 N NaOH for 20 minutes at 100°C (72). An extracellular polymer is present which appears distinctly different from that produced

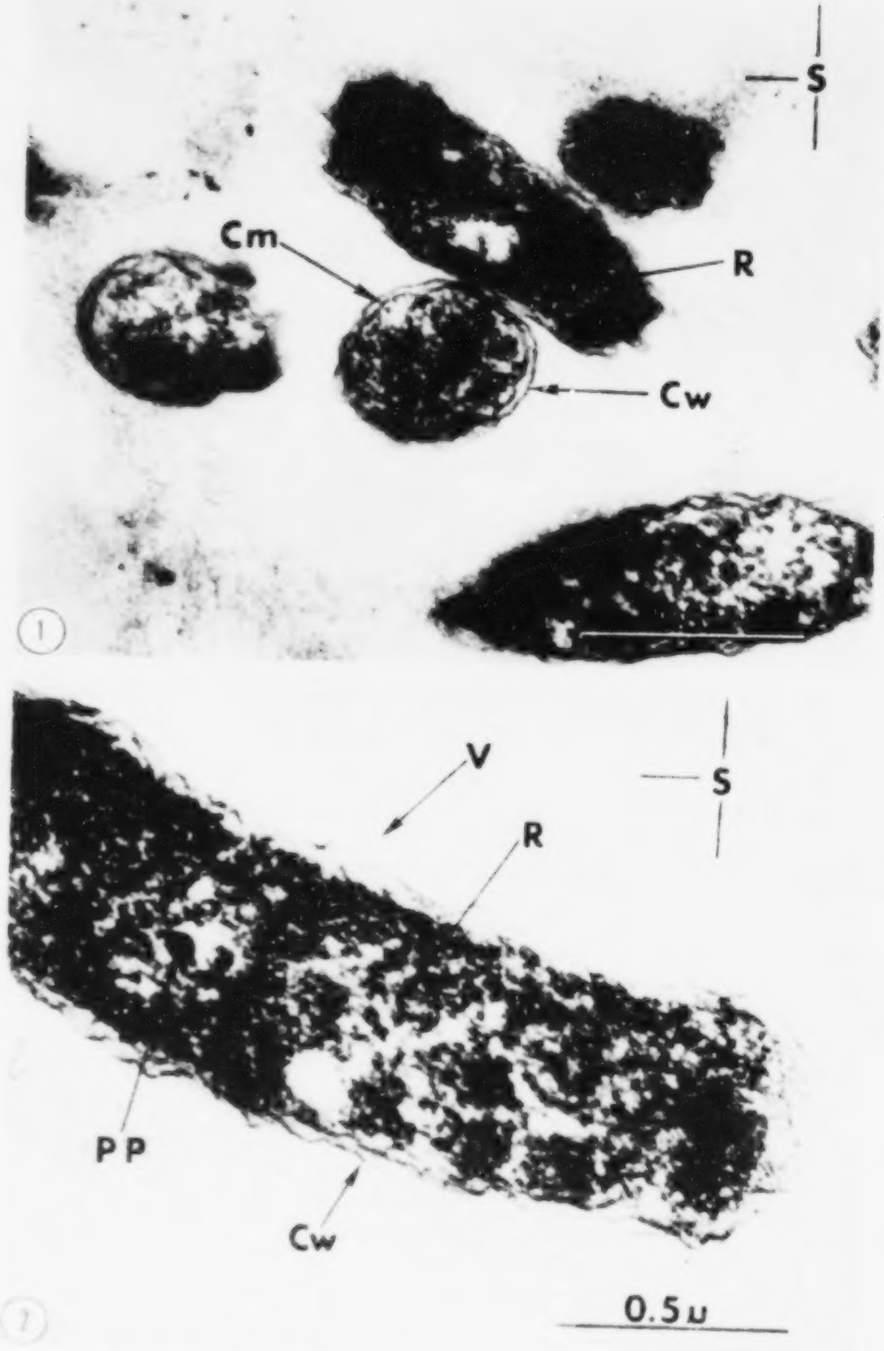


Figure 19. Thin sections of *Zoogloea ramigera* 115. (1) Thin section of 115 showing cell wall (Cw), cell membrane (Cm), ribosomes (R), and strands (S) of exocellular matrix. X64,000 (2) Thin section of 115. In addition to the above a polyphosphate granule (PP) and a distinct void (V) are present. The convoluted wall of 115 is quite in evidence. The diameter of the strands is 40-60 A. X67,000 Thin sections and photographs courtesy of Dr. R.M. Pfister

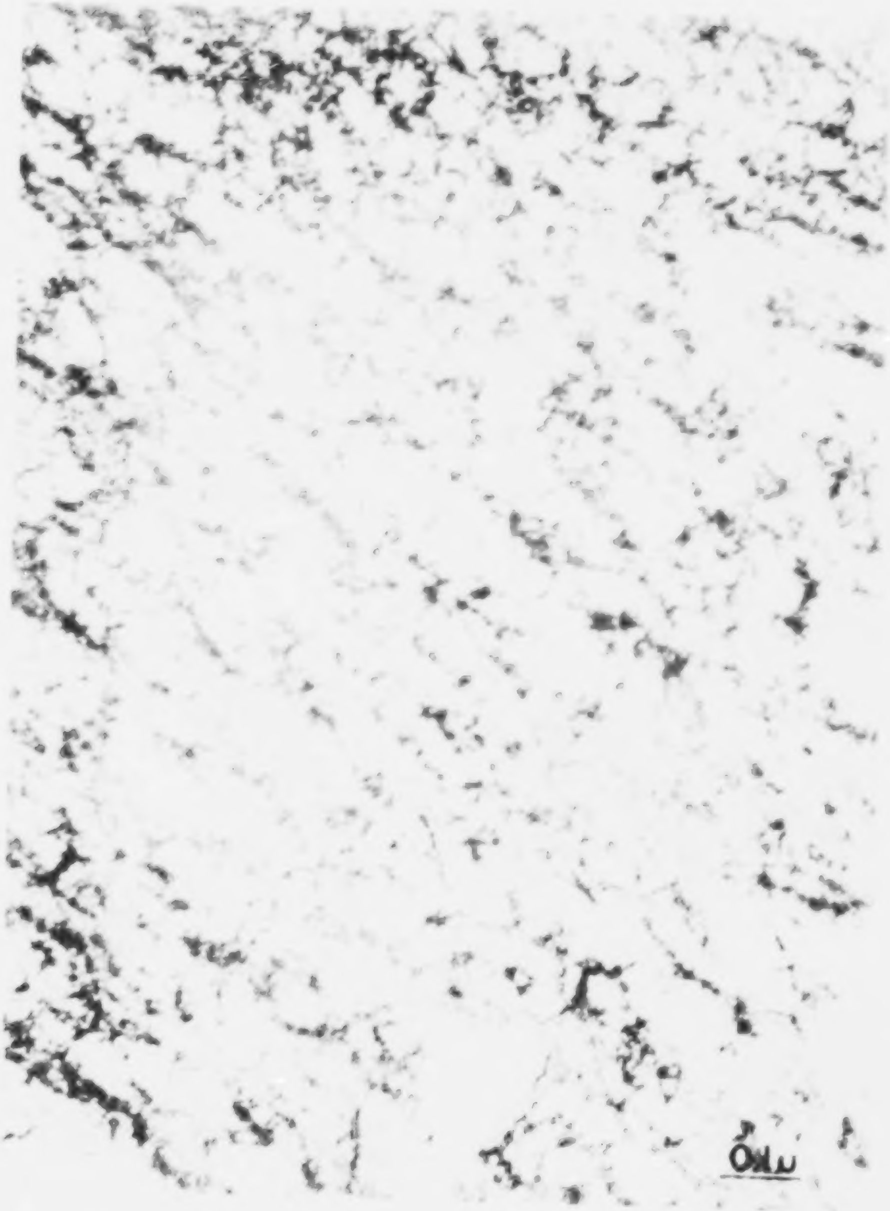


Figure 20. Purified extract of Zoogloea ramigera 115 exocellular matrix. Strands present are 20-50 A in diameter. X122,500 Photograph courtesy of Dr. R.M. Pfister

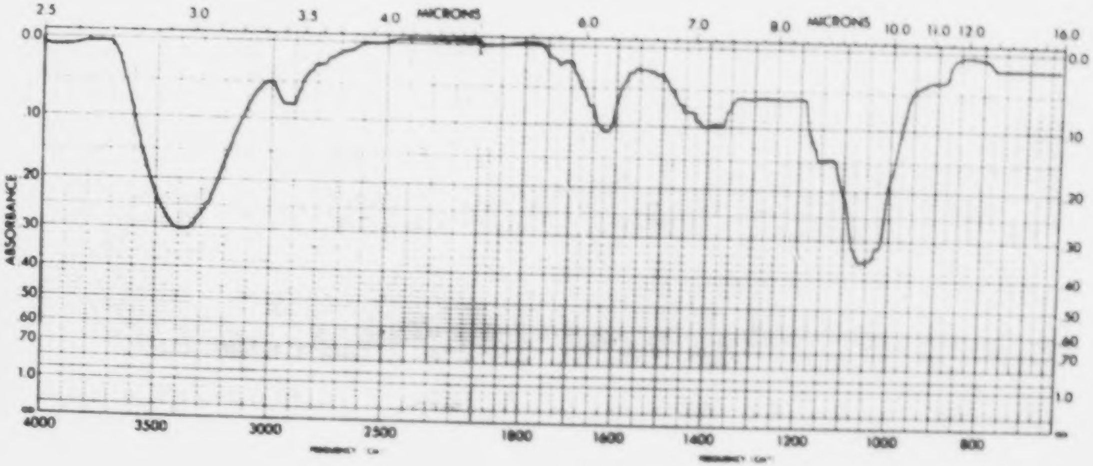


Figure 21. Infrared spectrum of purified exocellular matrix of Zoogloea ramigera 115

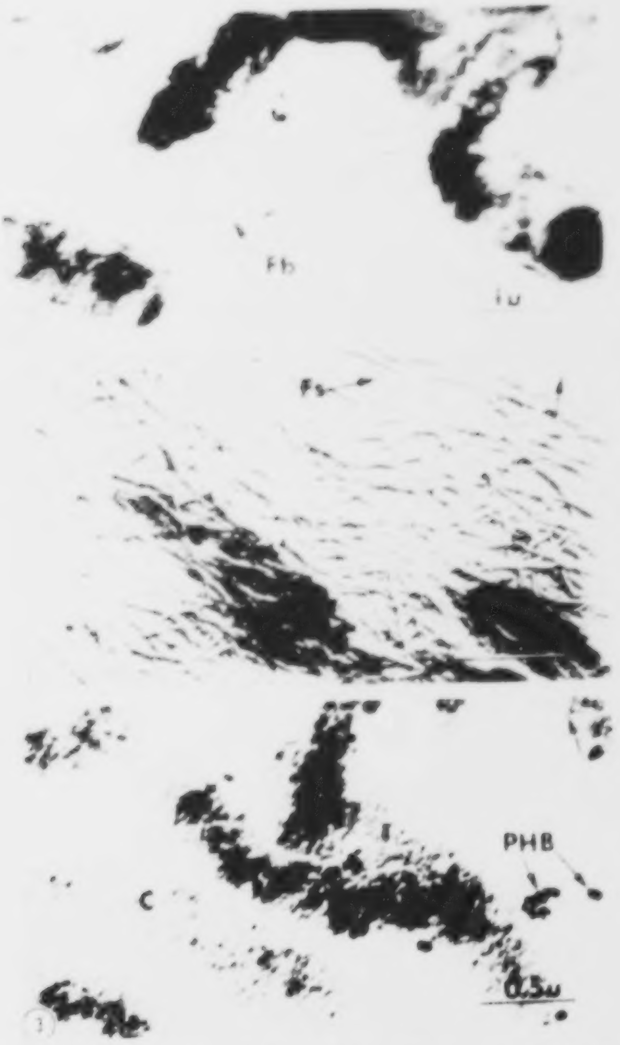


Figure 22. Comparison of Zoogloea ramigera I-16-M to 115 after treatment with NaOH. (1) Shadow-casting of I-16-M showing fibrils (Fb) and flattened cells (C), X19,500. (2) Another shadow-casting of I-16-M. Single fibrils (Fs) are 120-140 A in diameter, X22,700. (3) Shadow-casting of 115 showing remnants of cells (C). Dark granules are probably poly-beta-hydroxy-butyric acid (PHB) X41,200

by isolate 115 when treated in the same manner. No shadow-cast photographs of isolate 115 cells are presented because the 115 polymer is soluble in 1N NaOH. Figures 23-1 and 23-2 are frozen-etched preparations of the I-16-M isolate. Distinct extracellular strands can be seen attached to cells but the polymer configuration is different from that shown for 115 cells in Figs. 17 and 18.

The larger fibrils shown in Fig. 22 are approximately 12 to 14 nm in diameter and appear to be formed from elementary fibrils which are 2-5 nm in diameter. The large fibrils shown in Fig. 23 are about 40 nm in diameter. This suggested that in frozen etched preparations, material adheres to the fibrils, whereas it is removed when treated with 1N NaOH (Fig. 22). A granule which is presumed to be PHB is also present. Figure 24-1 shows PHB granules isolated from 115 cells and Fig. 24-2 shows more clearly the PHB granules in I-16-M cells.

Figure 25 compares individual cells and flocs of *Z. ramigera* I-16-M under brightfield and ultraviolet illumination.

Z. ramigera isolate I-16-M has been described as a nonzoogloeal matrix producing organism (27). Figure 7a is a photograph of an isolate I-16-M floc which has been stained with 1% aqueous crystal violet. No exocellular material was observed using this technique and many of the cells appeared to be free from the main clump of cells. When the isolate I-16-M flocs were examined in a wet mount without staining they also appeared as dispersed cells without an identifiable extracellular matrix as shown in Fig. 25-1. However, Fig. 25-2 shows that when the same flocs as shown in Fig. 25-1 are stained with the fluorescent dye Paper White BP and photographed through an ultraviolet microscope, the dye can be seen to be concentrated between the cells. This suggested the presence of substances which have a high affinity for the dye (e.g., cellulose or orther polysaccharides). Figures 25-3, 25-4, 25-5 and 25-6 show the same effect using larger flocs and lower magnification.

The association of the fluorescent dye with cell-flocs was quite rapid and wet mounts could be examined immediately after preparation. Fluorescence with I-16-M was initially quite intense, but gradually lessened during a 5 minute interval. *Z. ramigera* 115 fluoresced weakly when compared to I-16-M or particles of cellulose used as controls.

Several other isolates which have been partially described previously were also examined for the presence of extracellular polymers. *Pseudomonas* C-3 was examined by several techniques (Fig. 26) all of which showed the presence of fibrils surrounding the cells. Figure 26-3 is a shadow cast preparation and shows fibrils obtained by placing the cell-flocs in 1N NaOH for 24 hours at 28°C. Ghosts of several cells remain.

Fibrils extend outwardly (Fig. 26-1) as rays (f) from the cross sections. These rays may be analogous to the polymer matrix observed with frozen etched preparations of *Z. ramigera* isolate 115 and may represent a microcapsule surrounding the cells.

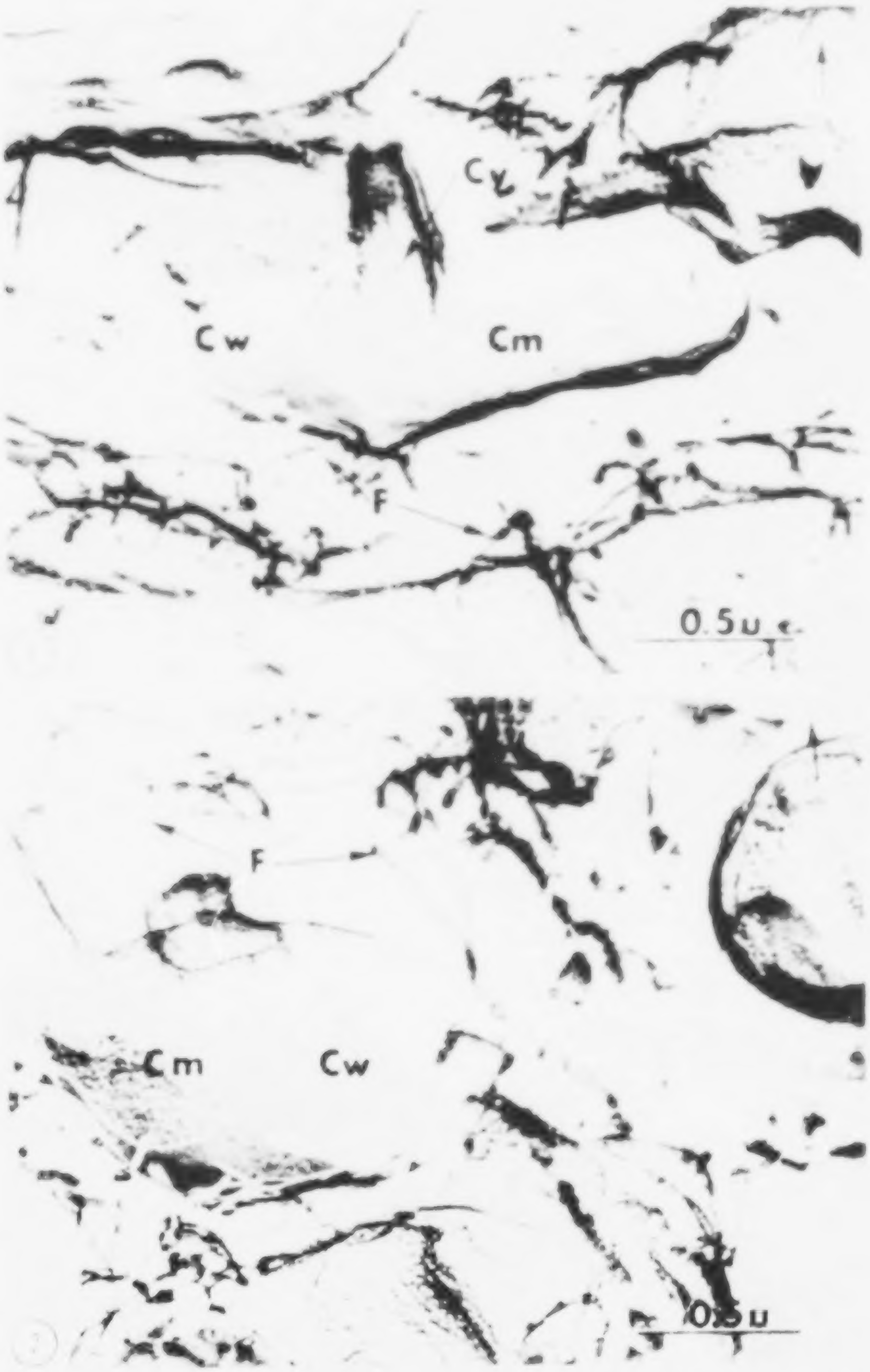


Figure 23. Fibrils of Zoogloea ramigera I-16-M. (1) Freeze-etching showing fibrils (F) around I-16-M. The cell wall(Cw), cytoplasmic membrane (Cm), and cytoplasm (Cy) are also present. X48,000 (2) Freeze-etching of I-16-M showing additional fibrils X48,000

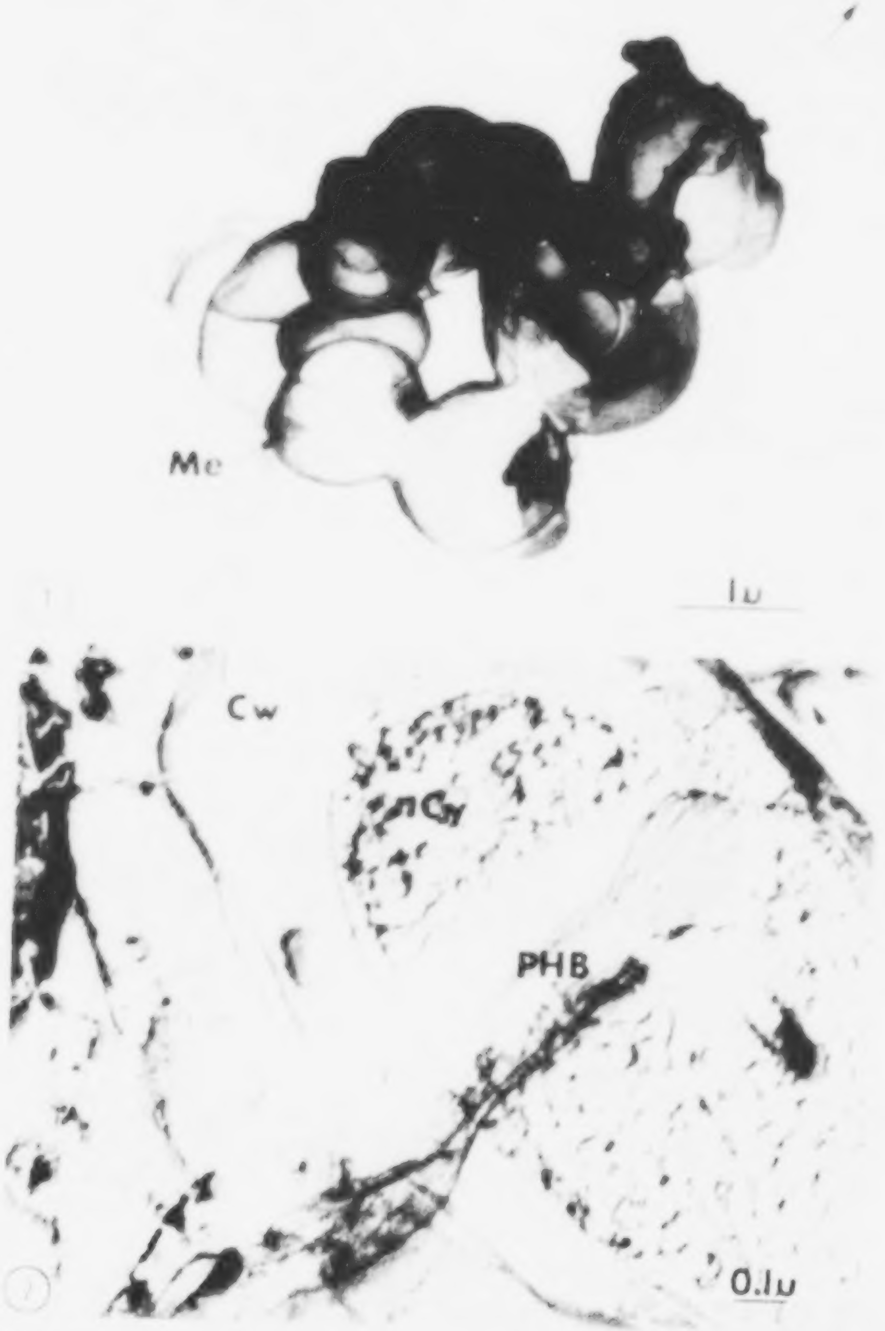


Figure 24. Poly-beta-hydroxybutyric acid granules (PHB) obtained from *Zoogloea ramigera* 115 and I-16-M. (1) Carbon replica of PHB granules obtained from freeze-dried preparation of *Zoogloea ramigera* 115. Granules possess a rough surface and perhaps a partial membrane covering (Me). X19,400 (2) Freeze-etching of *Zoogloea ramigera* I-16-M showing stretched PHB granules. The cell wall (Cw) and cytoplasm (Cy) are also visible X81,000

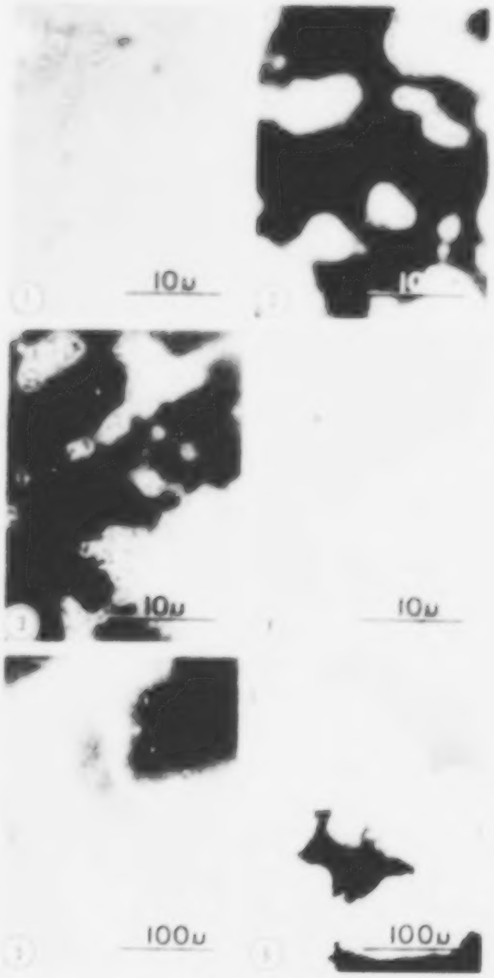


Figure 25. Brightfield and ultraviolet microscopy of Zoogloea ramigera I-16-M. (1) Individual cells viewed with white light, X2,000. (2) Individual cells viewed with ultraviolet light, X2,000. (3) Floc viewed with white light, X2,200. (4) Same floc viewed with ultraviolet light, X2,200. (5) and (6) other flocs viewed with ultraviolet light, (5) X240 and (6) X226. All cells and cell-flocs were photographed in the presence of Paper White BP, a fluorescent brightener

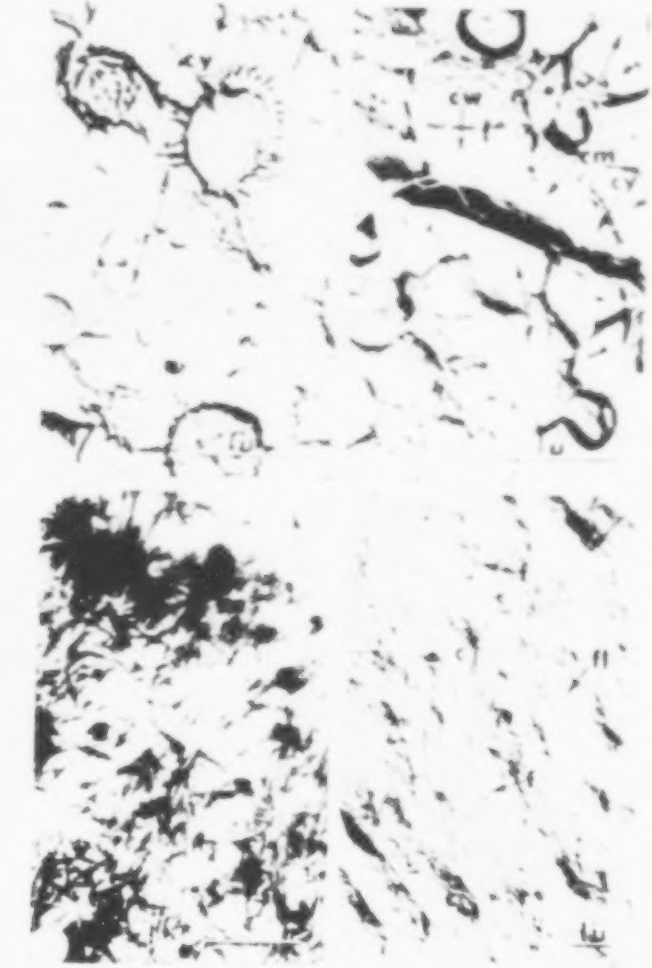


Figure 26. Freeze-etchings, shadow-casting, and carbon replica of Pseudomonas C-3. (1) Cross section as seen with freeze-etching. Rays (r) extending outward and cytoplasm (cy) are seen, X25,800. (2) Freeze-etching showing fibrils (f). In addition the cell wall (cw), cell membrane (cm), and cytoplasm (cy) are visible, X25,200. (3) Shadow-casting showing fibrils (f) and granules, probably PHB. The remnants of a cell (c) are seen, X39,900. (4) Carbon replica showing cells (c) and fibrils (f) intertwining around cells. Thicker fibrils, i.e., flagella (fl), are present, X9,000

Figure 26 shows an entire cell with elementary fibrils (f) adhering to the outer cell surface. The cell wall (cw), cell membrane (cm), and cytoplasm (cy) can be seen.

Figure 26-4 is a carbon replica of isolate C-3. Fibrils are quite common and crisscross over the cells. Thicker fibrils which may be flagella (fl) can be seen. Figures 26-1 and 26-2 are frozen etched preparations of isolate C-3.

Shadow cast preparations of other floc-forming bacteria also showed the presence of fibrils attached to cells. Figure 27-1 is a preparation of *Z. filipendula* isolate P-8-4 (26, 27, 41). Figure 27-2 is *Pseudomonas denitrificans* isolate P-95-5 (26, 27, 41). Figure 27-3 is an unidentified gram negative rod isolate C-22-4 (26, 27, 41). Figure 27-4 is a gram negative rod which had been identified as a *Z. ramigera* isolate Z-SC-38 (44).

The zoogloeal matrix which has been the primary basis for differentiating *Zoogloea* from other pseudomonads is composed of polymer strands. The "finger-like" projections appear to be built up from generally globular shaped packets of cells which adhere one to another. This is interpreted as being responsible for the flocculent habit of growth of this particular organism. The involvement of poly-beta-hydroxybutyric acid (PHB) as reported by Crabtree et al. (27) has not been ruled out but does not appear to play a direct role. This will be considered further in subsequent section of this report. Both 115 and I-16-M isolates produce large amounts of PHB under certain growth conditions.

Two sizes of polymer strands are shown in Figs. 17 and 18. The purified polymer (Fig. 20) has only one size strand which measures 2 to 6 nm in diameter. This suggests that the larger strand shown in Fig. 17 may have been induced by the formation of ice crystals during the freeze-etching procedure. The loss of pure water during sublimation could cause a concentration of the residual solution, leaving a eutectic mixture remaining between ice crystals. This mixture would tend to associate with the small strands causing them to appear larger.

The available chemical and spectroscopic data indicate that the polymer of isolate 115 is a polysaccharide; it is slightly soluble in water and solubility increases in alkaline solutions ($NH_4OH < NaOH$). Glucose and galactose are the only polymer components obtained from acid hydrolysis and susceptibility of the polymer to cellulase strongly suggests that the polymer resembles cellulose (i.e. contains 1, 4-beta-glycosidic bonds). Identification of the polymer as a type of cellulose must be made with caution because the crude cellulase enzyme may contain an unknown activity on the polysaccharide substrate. The polymer does not appear to be identical to the bacterial cellulose described by Muhlethaler (69) but has some resemblance to the cellulose fibrils shown by Gibson and Colvin (45). Further supporting evidence is being sought. Lack of susceptibility to either alpha or beta amylase indicates an absence of 1, 4-alpha-glycosidic bonds. A negative color response to iodine is also noted.

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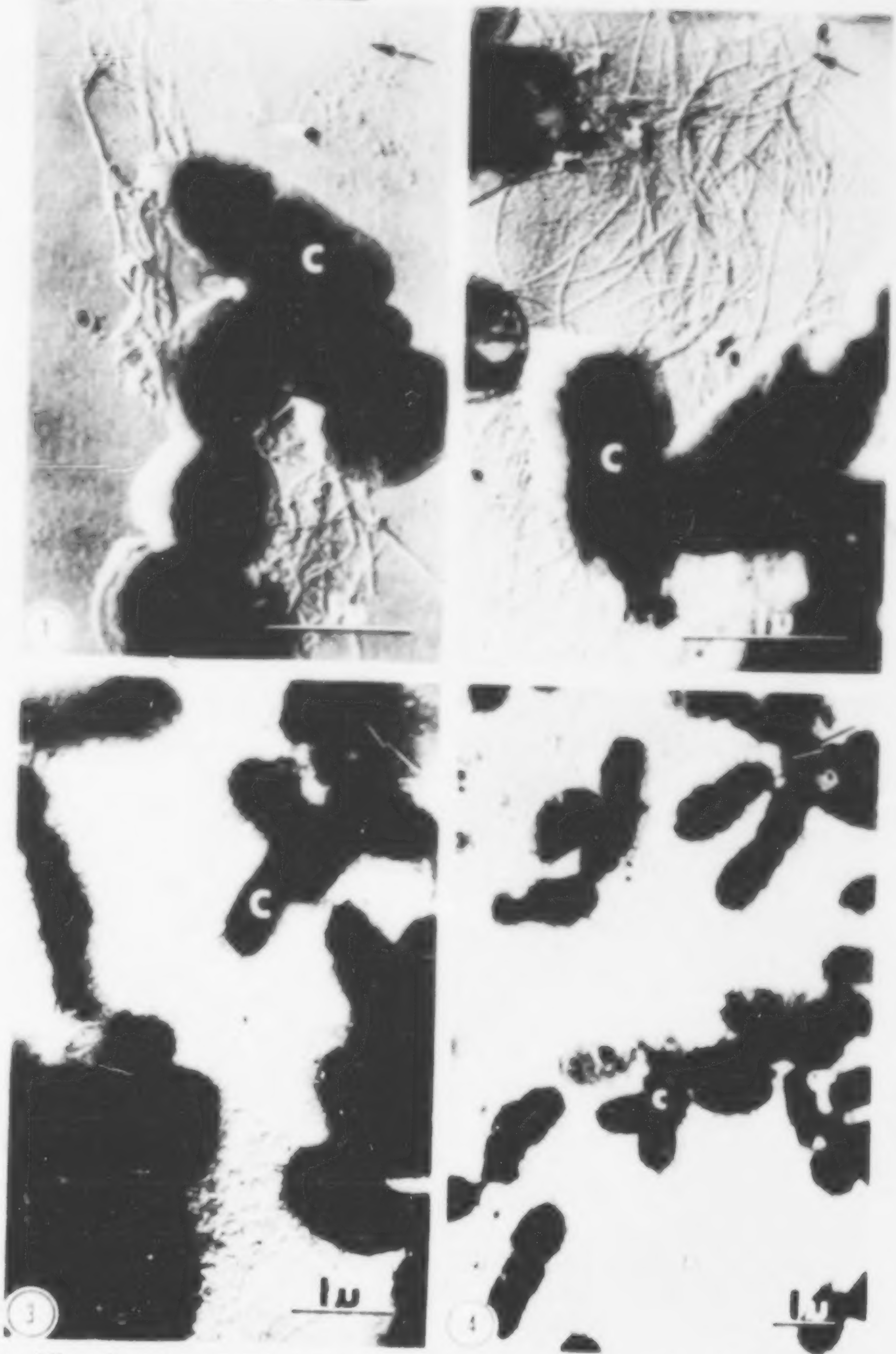


Figure 27. Shadow-casting of four floc-forming bacteria. (1) Zoogloea filipendula P-8-4, X21,000 (2) Pseudomonas dentrificans P-95-5, X23,500. (3) unidentified gram-negative rod C-22-4, X16,200. (4) Zoogloea ramigera Z-SC-38, X9,200. Cells (C) and fibrils are seen.

The exopolymer which surrounds the I-16-M isolate has not been isolated and purified so that comparisons to the polymer of isolate 115 must be made with caution. However, the difference in fine structure as well as different solubility properties in basic solutions suggests that the polymers are not identical, although the polymers from both 115 and I-16-M cells are susceptible to cellulase.

The polymer of isolate 115 appears to act as a polyelectrolyte, analogous to synthetic ion exchange resins or those used in gel filtration. It is probable that the matrix can serve a nutritional function for the cell by concentrating nutrients from solution. This could explain how organisms growing in extremely dilute nutritional aquatic habitats can accumulate essential nutrients. These same suggestions are applicable to the I-16-M isolate, which we conclude is a distinctly different organism than *Z. ramigera* isolate 115.

The possibility of cellulose synthesis by cells which do not produce acids raises some interesting taxonomic problems. If the generality of this result can be established it may be a definitive criterion which will aid in identifying organisms in the genus *Zoogloea*.

The data presented suggest that bacteria which have been isolated on the basis of their characteristic flocculent growth habit, all possess extracellular fibrillar polymers. Entanglement of cells among fibrils, or adsorption of cells to fibrils is a plausible explanation of the flocculation phenomenon. This conclusion supports the views of Tenney and Stumm (90), Busch and Stumm (13), Clark (23) and to some extent those of Finstein (38) and Heukelekian (50). The physical and chemical properties of the specific exocellular fibril polymers will determine the extent to which water is bound to the polymer and will also determine the solubility properties of the polymers and their affinity for stain. Therefore some bacterial polymers will appear as observable capsules whereas others will not.

The purified polymer from *Z. ramigera* isolate 115 was reported to have similar properties to synthetic polyelectrolytes (42) and flocculation of bacteria by synthetic polyelectrolytes would be analogous to the naturally occurring process. It can be further postulated that polyvalent ions can complex with functional groups on two different elementary fibrils thereby resulting in an effective bridge and alteration of the polymer charge. The association of extracellular polymers produced by one of the isolates (C-22-4) with sub-microscopic inorganic particles has been reported (76, See Fig. 46). We wish to point out the analogy of polymer bridges among bacterial cells.to similar polymer bridges among inorganic or synthetic materials (e.g. polystyrene latex balls) such as reported by Ries and Meyers (83).

Floc formation from other types of polymer should also be considered. Wessman and Miller (99) found that clumping of *Pasteurella pestis* was caused by the polymerization of extracellular nucleic acids that were excreted by the cells. The role of PHB reported by Crabtree et al. (26)

should also be further considered as an explanation of bacterial flocculation. (Also see section on polymer production and Fig. 41.)

One additional point concerns the taxonomic status of the floc-forming isolates described. All of the flocs were susceptible to cellulase and showed a positive hexose response within 15 to 120 min. Quantitative values are not presented here because the polymers were not always obtained in pure form and the quantitative variations probably reflect varying amounts of polymer surrounding individual cell isolates.

All of the isolates are polarly flagellated rods which do not resemble *Acetobacter xylinum* or *Sarcina ventriculi*. Few of the isolates are acid producers and most do not closely resemble *Gluconobacter* or *Acetomonas* in this regard. If the polymer around certain isolates proves to be cellulose, a taxonomic reappraisal would be in order.

Aquatic floc-forming organisms other than those investigated during this study have since been shown, in this laboratory, to produce extracellular polymer fibrils (e.g. *Azotobacter*, *Bacillus*, *Chromobacterium*, *Flavobacterium*) and the association of floc-formation with extracellular fibrils appears to be a valid generalization. Figure 28 is an electron micrograph of a frozen etched preparation of a gram positive spore forming aerobic rod (*Bacillus*) and serves to illustrate that bioflocculation is not limited to gram negative organisms. See also (34, 50).

One additional comment which should be made is that some bacteria tend to grow as a pellicle at an air-liquid interface. These surface films of bacteria may then settle and appear as flocs. It is probably without value to differentiate between pellicle formers and floc-formers in a discussion of bioflocculation since the net effect is quite similar. Figures 29A and 29B are electron micrographs of carbon replicas of an isolate [illegible] pellicle which shows the close packing of cells that grow at an air-water interface.

Concentration and Accumulation of Chemicals

Gross uptake of metallic ions. Results of these uptake studies are listed in Table 16. These experiments gave an indication of the extent which pregrown cells would concentrate and accumulate metal ions from solution in a non-growth situation. The 115 cell-flocs have a remarkable high affinity for Co^{+2}, Cu^{+2}, and Fe^{+2}, and somewhat less for Ni^{+2}.

Values obtained after contact periods of 2 and 3 days indicate that no further uptake of ions occurs beyond 18 hours. No data were obtained for time intervals less than 18 hours in this experiment. Comparison of data obtained using 115 cells to data from I-16-M cells shows that approximately twice as much metal ion is accumulated by the zoogloeal producing strain. This suggests that about half the ion uptake is concentrated in the zoogloeal matrix surrounding the cells if it is assumed that 115 has a matrix and I-16-M has none. However, Figs. 22, 23 and 25 illustrated a matrix around I-16-M but the extent has not been quantitated.

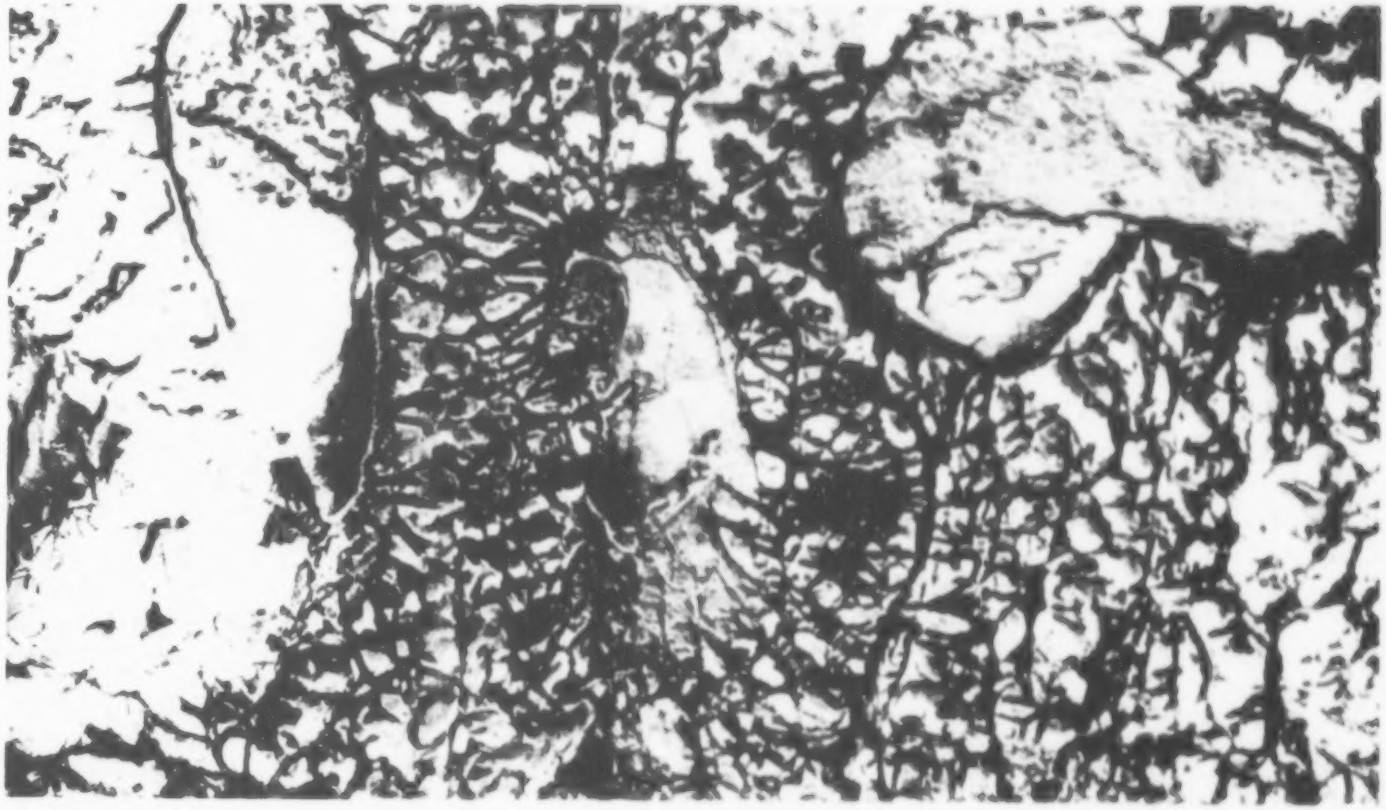


Figure 28. Electromicrograph of a frozen etched preparation of a gram positive *Bacillus* species showing that bioflocculation is not limited to gram negative organisms. X37,000

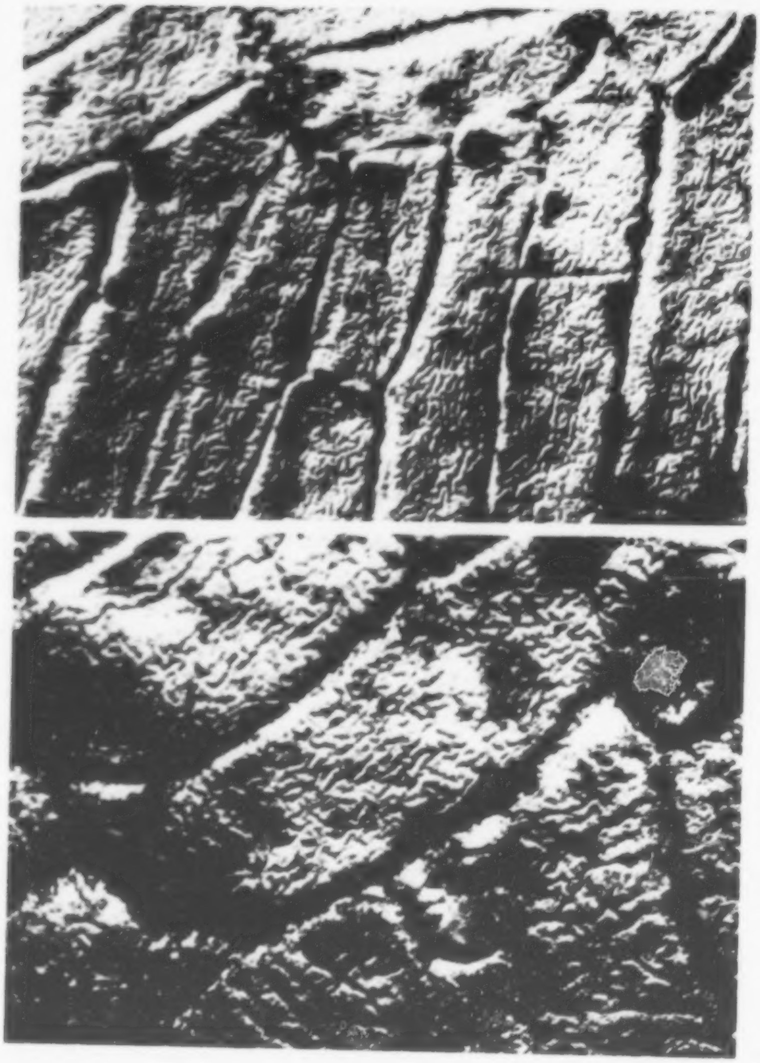


Figure 29. Electron micrographs of carbon replicas of surface pellicles of isolate C-3 showing closely adhering cells; A, X30,000, B, X50,000

TABLE 14. Values showing initial concentration of metallic ion in solution ($\mu g/ml$) and concentration after shaking for 18 hours in presence of Zoogloea ramigera 115 (0.1 g, dry wt) or Zoogloea ramigera I-16-M (0.12 g, dry wt)

	Cobalt as $CoCl_2 \cdot 6H_2O$			Copper as $CuCl_2 \cdot 2H_2O$			Iron as $FeCl_3 \cdot 6H_2O$			Nickel as $NiCl_2 \cdot 6H_2O$		
Initial Concentration Zoogloea ramigera 115	50	240	500	90	400	800	34	180	350	50	250	500
Concentration after 18 hours	1.3	2.2	3.5	73	97	121	0.5	3.2	4.3	41	190	250
% removal	97.5	99.1	99.3	18.9	75.3	84.9	98.5	98.2	98.8	18	24	50
Total removed, mg/100 mg cells (dry wt)	2.4	11.9	24.8	0.8	15.1	34	16.7	8.8	17.3	0.4	3	12.5
Zoogloea ramigera I-16-M Concentration after 18 hours	2.8	64	310	4.7	150	420	0.3	1.1	187	32.6	164	376
% removal	94.5	73.3	38	94.8	62.5	47.5	99.1	99.4	46.6	34.8	34.4	24.8
Total removed, mg/100 mg cells (dry wt)	2.3	8.8	9.5	4.3	12.5	19	16.8	9	8.2	0.9	4.3	6.2

The uptake of various combinations of cations by Z. ramigera 115 was also studied. As shown in Table 14, almost 100% of Co^{+2} and Fe^{+3} were absorbed by 115 after an 18 hour contact period. Similar experiments calculated in micromoles (μmoles) give analogous results (Table 15). The theoretical value (μmoles added) is the sum of all cations placed in solution for a particular test. The number of μmoles removed from solution is the quantity removed by the cell-flocs. When two or three ions were added (Tables 16 and 17), the greatest accumulation usually occurred in the second concentration of each complete set of ions. Although the first concentration of each set of ions might be thought to absorb all of the ions present because of the availability of sites, the low values suggest this is unlikely. The decrease in values obtained with the third concentration suggests a loss from oversaturation. The order of absorption is $Fe^{+3} > Co^{+2} > Cu^{+2} > Ni^{+2}$.

Uptake of metal ions by I-16-M indicates that cells in the absence of zoogloeal matrix can concentrate a considerable amount of metal ion. However, the ions may accumulate, at least in part, on the polymer strands shown to be present around I-16-M cells.

The cells accumulated 34% of their total weight of Cu^{+2} and approximately 25% of Co^{+2}. This precludes use of this type of experiment to determine metal uptake by growing cells vs. time. That is, no accurate cell weight determinations could be made in the presence of relatively high concentrations of ions. Cell counting techniques are not feasible when the cells are embedded in a zoogloeal matrix. More precise data pertaining to metal uptake by both growing and non-growing cells were obtained using the radioisotope $Zn^{65}Cl_2$.

Accumulation of Zn^{+2} by actively growing cells. Figure 30A shows the accumulation of Zn^{65} in the presence of 10 μg carrier Zn^{+2}/ml of growth by Z. ramigera cell-flocs over an 11 day period. Loss of Zn^{+2} from culture medium is also shown. Loss of Zn^{+2} from supernatant correlates with uptake of Zn^{+2} by cell-flocs when total counts/min are compared. Accumulation of Zn^{+2}/0.2 g cell-flocs has also been plotted. This curve incicates that the rate of Zn^{+2} uptake is constant with time from the 6th to the 11th day. Total uptake during this time increases because of the net increase in cell-floc weight. Total weight increase of cell-floc vs. time is shown in Fig. 30B. Weight of cell-floc is emphasized because cell numbers do not increase in proportion to weight over the entire 11 day period. The decrease in Zn^{+2} associated with the cell-floc fraction between the 5th and 7th day suggests a shift in metabolism of the organism. This could possibly reflect either a conversion from cell reproduction to zoogloeal matrix synthesis or accumulation and utilization of poly-beta-hydroxybutyric acid within the cells. The release of Zn^{65} from cell-floc back into growth medium during the 5-7 day interval appears to be real. Similar curves were obtained with Zn^{65} alone or with Zn^{65} plus 1 μg/ml carrier Zn^{65} plus 1 μg/ml carrier Zn^{+2}.

TABLE 15. Gross uptake of metallic ions after 18 hour contact with pre-grown *Zoogloea ramigera* 115 cell-flocs

Cations	Fe^{+3}			Co^{+2}		
Series	1	2	3	1	2	3
Theoretical (μmoles added)	0.63	3.15	6.30	0.85	4.25	8.5
μmoles removed from solution	0.63	3.15	6.30	0.85	4.25	8.5
% of theoretical uptake	100	100	100	100	100	100

Cations	Cu^{+2}					
Series	1		2		3	
Theoretical (μmoles added)	1.26		6.30		12.60	
μmoles removed from solution	0.1	-	4.78	4.76	10.53	11.9
% of theoretical uptake	13	0	76	76	83	95

Cation	Ni^{+2}								
Series		1			2			3	
Theoretical (μmoles added)		0.85			4.27			8.54	
μmoles removed from solution	0.15	0.17	0.51	1.02	2.39	2.04	4.27	6.65	6.65
% of theoretical uptake	18	20	60	24	56	48	50	78	78

TABLE 16. Gross uptake of various combinations of metallic ions (two cations) after 18 hour contact with pre-grown Zoogloea ramigera 115 cell-flocs

Concentration	Cations	Theoretical (μmoles added)	μmoles removed from solution	% of theoretical uptake
1	Fe^{+3}	0.63	0.63	100
	Ni^{+2}	0.85	0.25	29
	$Fe^{+3}+Ni^{+2}$	1.48	0.88	59
2	Fe^{+3}	3.15	3.15	100
	Ni^{+2}	4.27	4.21	99
	$Fe^{+3}+Ni^{+2}$	7.42	7.36	99
3	Fe^{+3}	6.30	6.30	100
	Ni^{+2}	8.54	1.28	15
	$Fe^{+3}+Ni^{+2}$	14.84	7.58	51
1	Fe^{+3}	0.63	0.63	100
	Cu^{+2}	1.26	0.24	19
	$Fe^{+3}+Cu^{+2}$	1.89	0.87	46
2	Fe^{+3}	3.15	3.15	100
	Cu^{+2}	6.30	6.03	96
	$Fe^{+3}+Cu^{+2}$	9.45	9.18	97
3	Fe^{+3}	6.30	6.30	100
	Cu^{+2}	12.60	5.43	43
	$Fe^{+3}+Cu^{+2}$	18.90	11.73	62
1	Co^{+2}	0.85	0.76	89
	Ni^{+2}	0.85	0.29	34
	$Co^{+2}+Ni^{+2}$	1.70	1.05	62
2	Co^{+2}	4.25	4.23	99
	Ni^{+2}	4.27	3.72	87
	$Co^{+2}+Ni^{+2}$	8.52	7.95	93
3	Co^{+2}	8.50	7.62	90
	Ni^{+2}	8.54	-	-
	$Co^{+2}+Ni^{+2}$	17.04	7.62	45

TABLE 16. Continued

Concentration	Cations	Theoretical (μmoles added)	μmoles removed from	% of theoretical uptake
1	Co^{+2}	0.85	0.74	87
	Fe^{+3}	0.63	0.63	100
	$Co^{+2}+Fe^{+3}$	1.48	1.37	93
2	Co^{+2}	4.25	3.98	94
	Fe^{+3}	3.15	3.15	100
	$Co^{+2}+Fe^{+3}$	7.40	7.13	96
3	Co^{+2}	8.50	1.23	14
	Fe^{+3}	6.30	6.30	100
	$Co^{+2}+Fe^{+3}$	14.80	7.53	51
1	Co^{+2}	0.85	0.78	92
	Cu^{+2}	1.26	0.18	14
	$Co^{+2}+Cu^{+2}$	2.11	0.96	46
2	Co^{+2}	4.25	4.06	96
	Cu^{+2}	6.30	5.66	90
	$Co^{+2}+Cu^{+2}$	10.55	9.72	92
3	Co^{+2}	8.50	4.42	52
	Cu^{+2}	12.60	10.22	81
	$Co^{+2}+Cu^{+2}$	21.10	14.64	69
1	Cu^{+2}	1.26	0.22	17
	Co^{+2}	0.85	0.82	97
	$Cu^{+2}+Co^{+2}$	2.11	1.04	52
2	Cu^{+2}	6.30	4.80	76
	Co^{+2}	4.25	4.15	98
	$Cu^{+2}+Co^{+2}$	10.55	8.95	85
3	Cu^{+2}	12.60	12.20	97
	Co^{+2}	8.50	7.82	92
	$Cu^{+2}+Co^{+2}$	21.10	20.02	95

TABLE 16. Continued

Concentration	Cations	Theoretical (μmoles added)	μmoles removed from	% of theoretical uptake
1	Cu^{+2}	1.26	0.18	14
	Ni^{+2}	0.85	0.24	28
	$Cu^{+2}+Ni^{+2}$	2.11	0.42	20
2	Cu^{+2}	6.30	5.45	87
	Ni^{+2}	4.27	1.25	29
	$Cu^{+2}+Ni^{+2}$	10.57	6.70	64
3	Cu^{+2}	12.60	9.84	78
	Ni^{+2}	8.54	-	-
	$Cu^{+2}+Ni^{+2}$	21.14	9.84	47

TABLE 17. Gross uptake of various combinations of metallic ions (three cations) after 18 hour contact with pre-grown *Zoogloea ramigera* 115 cell-flocs

Concentration	Cations	Theoretical (μmoles added)	μmoles removed from solution	% of theoretical uptake
1	Co^{+2}	0.85	0.73	86
	Fe^{+3}	0.63	0.63	100
	Ni^{+2}	0.85	0.34	40
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	2.33	1.70	73
2	Co^{+2}	4.25	4.11	97
	Fe^{+3}	3.15	3.15	100
	Ni^{+2}	4.27	2.90	68
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	11.67	10.16	87
3	Co^{+2}	8.50	2.54	30
	Fe^{+3}	6.30	6.30	100
	Ni^{+2}	8.54	1.70	20
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	23.34	10.54	45
1	Co^{+2}	0.85	0.75	88
	Fe^{+3}	0.63	0.63	100
	Ni^{+2}	0.85	0.16	19
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	2.33	1.54	66
2	Co^{+2}	4.25	4.22	99
	Fe^{+3}	3.15	3.15	100
	Ni^{+2}	4.27	3.58	84
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	11.67	10.95	94
3	Co^{+2}	8.50	8.32	98
	Fe^{+3}	6.30	6.30	100
	Ni^{+2}	8.54	5.98	70
	$Co^{+2}+Fe^{+3}+Ni^{+2}$	23.34	20.60	88

TABLE 17. Continued

Concentration	Cations	Theoretical (μmoles added)	μmoles removed from solution	% of theoretical uptake
1	Fe^{+3}	0.63	0.63	100
	Ni^{+2}	0.85	0.42	49
	Cu^{+2}	1.26	0.54	43
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	2.74	1.59	58
2	Fe^{+3}	3.15	3.15	100
	Ni^{+2}	4.27	0.85	20
	Cu^{+2}	6.30	5.54	88
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	13.72	9.54	70
3	Fe^{+3}	6.30	6.30	100
	Ni^{+2}	8.54	0.68	8
	Cu^{+2}	12.60	5.71	45
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	27.44	12.69	46
1	Fe^{+3}	0.63	0.63	100
	Ni^{+2}	0.85	0.16	19
	Cu^{+2}	1.26	0.76	60
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	2.74	1.55	57
2	Fe^{+3}	3.15	3.15	100
	Ni^{+2}	4.27	2.78	65
	Cu^{+2}	6.30	5.67	90
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	13.72	11.60	85
3	Fe^{+3}	6.30	6.30	100
	Ni^{+2}	8.54	2.58	30
	Cu^{+2}	12.60	11.40	90
	$Fe^{+3}+Ni^{+2}+Cu^{+2}$	27.44	20.28	74

TABLE 17. Continued

Concentration	Cations	Theoretical (µmoles added)	µmoles removed from solution	% of theoretical uptake
1	Co^{+2}	0.85	0.73	86
	Cu^{+2}	0.26	0.76	60
	Fe^{+3}	0.63	0.63	100
	$Co^{+2}+Cu^{+2}+Fe^{+3}$	2.74	2.12	77
2	Co^{+2}	4.25	4.15	98
	Cu^{+2}	6.30	5.48	87
	Fe^{+3}	3.15	3.15	100
	$Co^{+2}+Cu^{+2}+Fe^{+3}$	13.70	12.78	93
3	Co^{+2}	8.5	6.57	77
	Cu^{+2}	12.6	12.20	97
	Fe^{+3}	6.3	6.30	100
	$Co^{+2}+Cu^{+2}+Fe^{+3}$	27.4	25.07	92
1	Co^{+2}	0.85	0.71	84
	Cu^{+2}	1.26	0.18	14
	Fe^{+3}	0.63	0.63	100
	$Co^{+2}+Cu^{+2}+Fe^{+3}$	2.74	1.52	56
2	Co^{+2}	4.25	3.91	92
	Cu^{+2}	6.30	3.67	58
	Fe^{+3}	3.15	3.15	100
	$Co^{+2}+Cu^{+2}+Fe^{+3}$	13.70	10.73	78
3	Co^{+2}	8.5	2.12	25
	Cu^{+2}	12.6	10.22	81
	Fe^{+3}	6.3	6.30	100
	$Co^{+2}+Cu^{+2}Fe^{+3}$	27.4	18.64	68

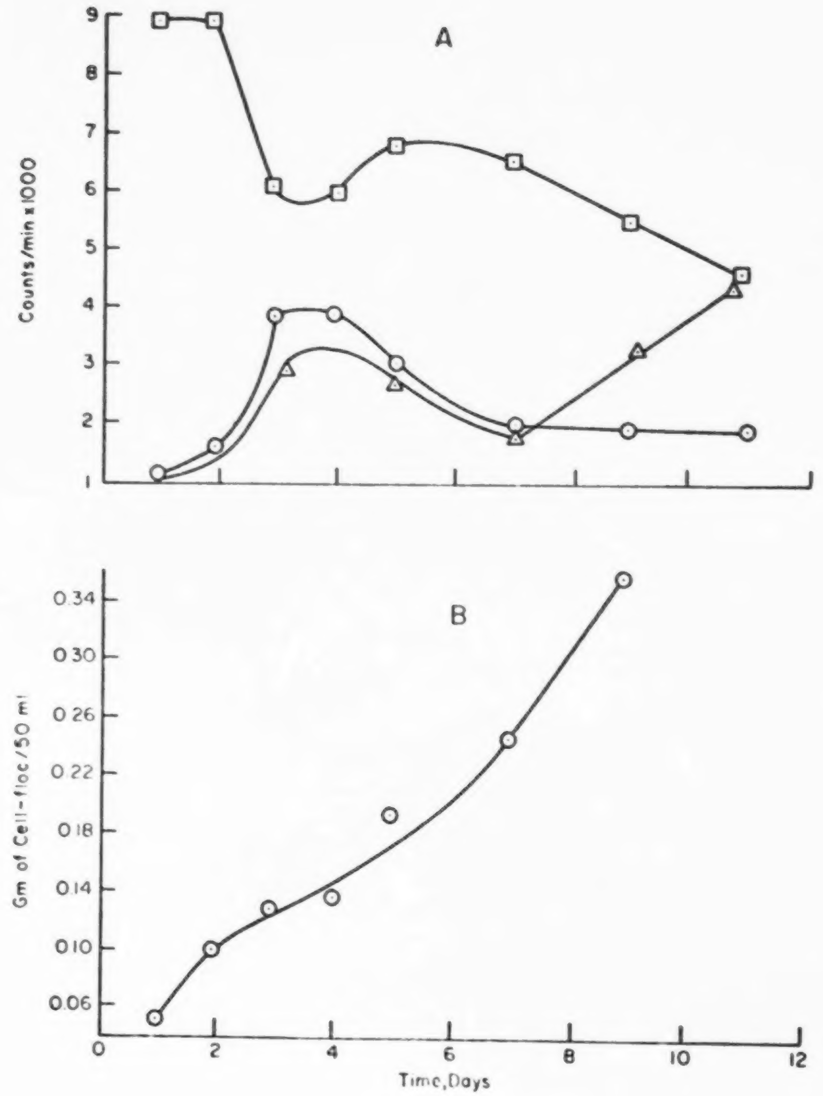


Figure 30. Accumulation of Zn^{+2} by *Zoogloea ramigera* 115 during growth (A) Curve showing counts per minute per 50 ml of growth medium supernatant vs. time (⊡); counts per minute per 0.2 g of cell-floc vs. time (⊙); counts per minute per total amount of cell-floc accumulated during growth period (△). (B) Total weight of cell-floc accumulation per 50 ml vs. time

Figure 31A shows the accumulation of Zn^{65} in the presence of 10 μg carrier Zn^{+2}/ml of growth medium by Z. ramigera I-16-M cell-flocs over a 9 day interval. Loss of Zn^{+2} from culture medium is also shown. Loss of Zn^{+2} from supernatant correlates with uptake of Zn^{+2} by cell-flocs when total counts/min are compared. Total weight increase of cell-floc vs. time is shown in Fig. 31B.

Counts/min obtained after one day of incubation are not of significance because of the small quantity of total cell-floc. Therefore, it is not justifiable to attribute the decrease on the second day to a growth lag. Total cell-floc weight does not continually rise as with 115, but recedes slowly after the second day. The total coun/min/0.2 g cells peaks at 4-5 days. The count rate averages 1000 counts/min higher than 115 under similar conditions. A comparison with gross uptake studies therfore indicates that while I-16-M can concentrate Zn^{+2} in a growth-rate uptake study, massive doses are not absorbed quickly or bound as tightly as with 115.

Turbidity of the TSB medium containing I-16-M gradually increases after the second day. The same is true of medium containing 115. The cell-flocs do not, however, lose as much Zn^{+2} with time as does the 115. In addition the weight does not increase. Similar curves were obtained with Zn^{65} alone or with Zn^{65} plus 1 μg/ml carrier Zn^{+2}.

Uptake of Zn^{+2} after pregrowth of culture. Figure 32 presents a curve showing Zn^{65} accumulation (no carrier) by 4 day pregrown 115 cell-flocs. Although the uptake curves on 1 day, 3 day, and 4 day cell-flocs were similar, the absolute values varied. This suggests that the affinity of cells for metal ions varies with growth state or physiological state of the organism. For example, 1 day cell-flocs showed an average of 1400 counts/min/0.2 g cells (dry weight) after 4 to 6 hours; 3 day cell-flocs had an average of 2750 counts/min/0.2 g cells after the same time. This indicates that actively growing cells (24 hour culture) have approximately half the affinity for Zn^{+2} accumulation that 4 day cells have. This is in general agreement with data presented in Fig. 30A.

Chemical oxygen demand. Figure 33 is a plot of supernatant vs. time of growth (Standard Methods, 1960) and serves to indicate net oxidation of nutrients by Z. ramigera 115. The curve shows a rapid decrease in the quantity of oxidizable material during the first two days. A minimum is reached, followed by a gradual increase in the quantity of oxidizable material in the supernatant. It might be noted that an inversion of this curve is somewhat analogous to a growth curve.

An interesting contrast between this graph and the net cell-floc synthesizing curve shown in Fig. 30 is the increase of cell-floc weight shown in curve B, in contrast to a plateauing of oxidizable materials in this graph. Results based solely on curve B would indicate that the cells continue to grow throughout the incubation period. However, the cells are no longer viable. This might suggest that the constituents in the medium are already in an oxidized form and could account for lack of further decrease in the chemical oxygen demand.

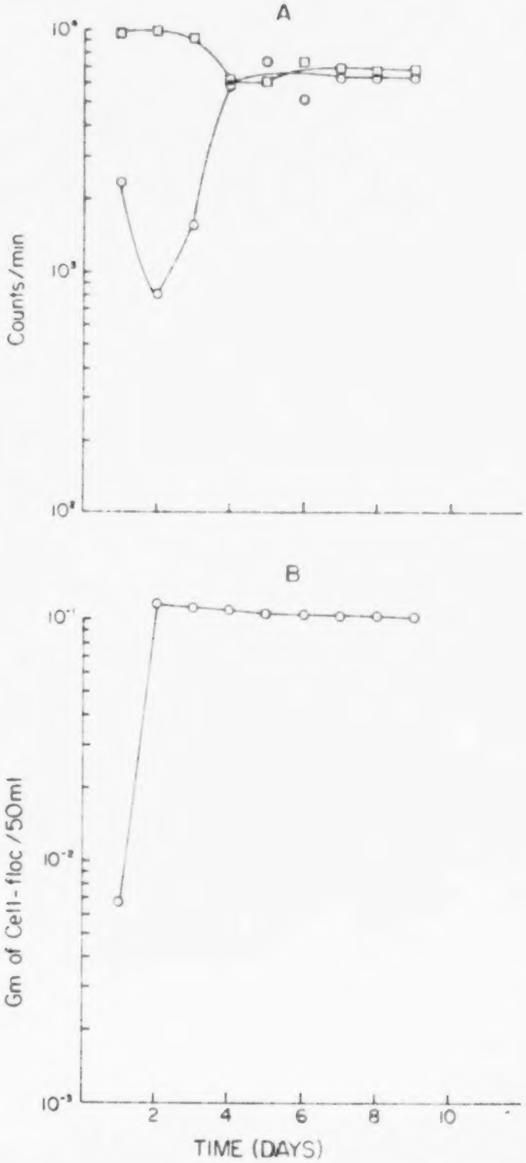


Figure 31. Accumulation of Zn^{+2} by Zoogloea ramigera I-16-M during growth. (A) Curve showing counts per minute per 50 ml of growth medium supernatant vs. time (▣); counts per minute per 0.2 g of cell-floc vs. time (⊙). (B) Total weight of cell-floc accumulation per 50 ml vs. time.

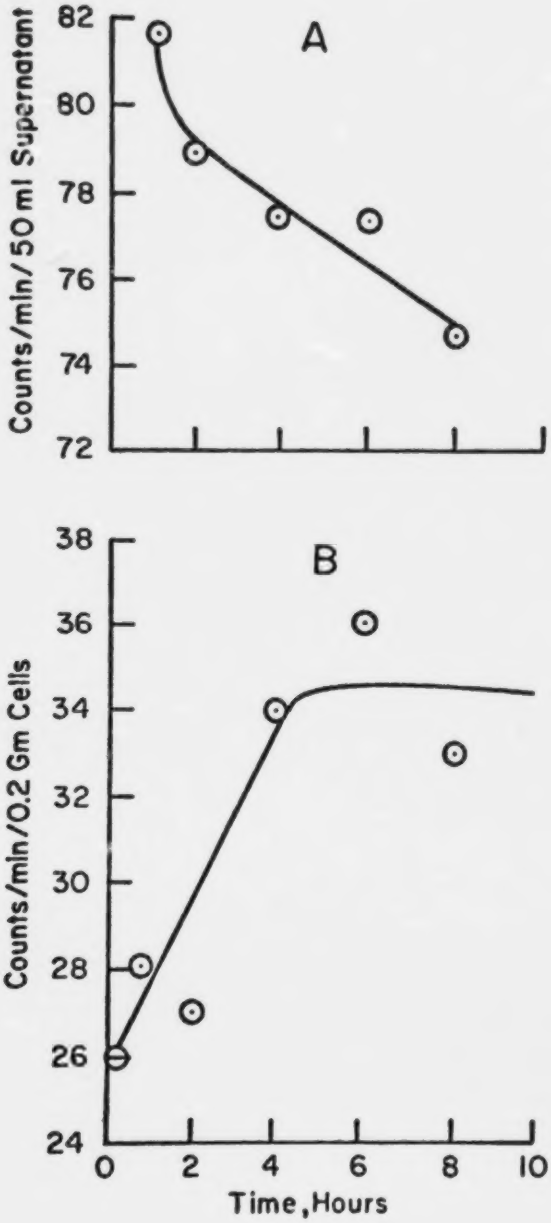


Figure 32. Accumulation of Zn^{65} by four day culture of Zoogloea ramigera 115 cell-flocs. (A) Counts per minute per 50 ml of growth medium supernatant (X100). (B) Counts per minute per 0.2 g dry weight of cell-floc (X100).

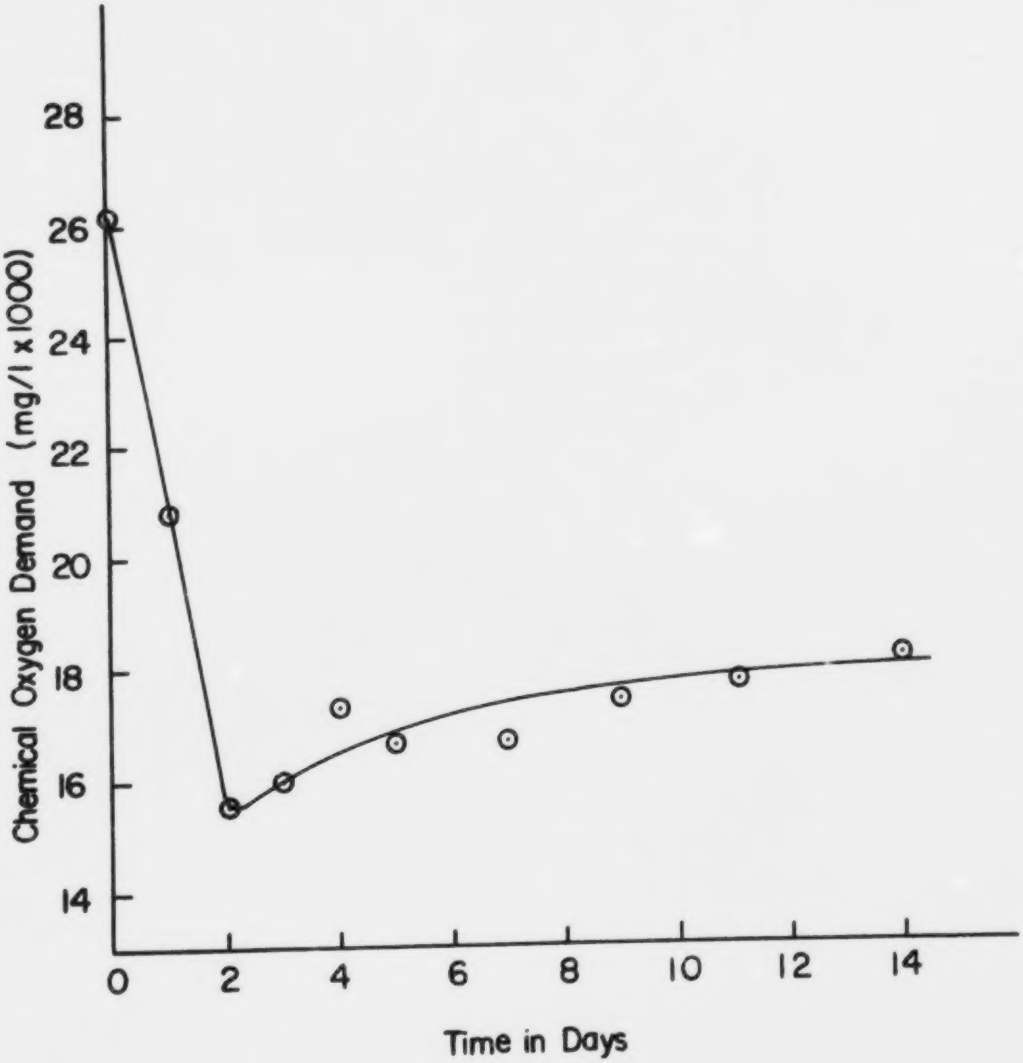


Figure 33. Chemical oxygen demand of medium containing _Zoogloea ramigera_ 115 during a fourteen day interval

Accumulation of Zn^{+2} at 21° and 28°C. A very rapid uptake of Zn^{65} plus 10 μg/ml carrier occurred in the presence of 4 day old Z. ramigera 115 at both temperatures (Fig. 34). However, at 28°C there was greater total uptake. It is observable that the difference in quantity of Zn^{+2} accumulated is related to the production of zoogloeal matrix. That is, little or no matrix is synthesized at 21°C and less Zn^{+2} is accumulated.

Interrelationship between the Zoogloeal matrix and Zn^{65} absorption. Similar results were obtained in experiments where Zn^{65} was added at the time of inoculation of culture, i.e., zero time, and after 3, 4, or 5 days of growth of the culture. Sixty to seventy percent of the Zn^{65} label remained in the supernatant. Approximately 30-40% of the label was absorbed by the cell-flocs. When the cells were separated from matrix the amount of Zn^{65} in the matrix represented a three to four-fold concentration over that observed with cells. The decrease of Zn^{65} in the cell-flocs on day 5 is similar to the decrease noted previously in Fig. 30A. These results are summarized in Fig. 35.

Uptake of Zn^{+2} by a crude extract. Uptake studies were performed with a crude extract of Z. ramigera 115 exocellular polymer plus Zn^{65} and either 10 or 100 μg/ml carrier. A sharp uptake of Zn^{+2} occurred, followed by a plateau (Fig. 36). Almost a ten-fold difference exists between the plots (the lower the quantity of carrier Zn^{+2}, the higher the count rate) which reinforces the postulation that most of the ion is absorbed by the matrix.

Acid Mine Water

In a series of experiments designed to determine the capacity of polymer to adsorb Fe^{+3} from acid mine water, 34.5 g (wet weight) of cell-floc-gel was exposed to 50 ml of $FeCl_3$ solution (125 μg Fe/ml) and allowed to shake for 3 hours. The floc was separated from the supernatant and fresh $FeCl_3$ solution was added and the procedure repeated, etc. A total of 16.8 mg Fe^{+3} was adsorbed by 34.5 g (wet weight) of cell-floc which is equivalent to about 0.3 g dry weight. Other experiments have shown that 90-98% of the cell-floc weight removed by centrifugation is due to extractable extracellular polysaccharide, after cultures have reached a stationary growth phase (e.g. 84 hours). This implies that the polysaccharide adsorbs over 100 times its own weight of water. Conversely about 1% of the cell-floc wet weight is due to polymer dry weight under experimental conditions.

Table 18 presents data showing adsorption of various mine water ions by the cell-floc present in 50 ml flasks (average of about 33.0 g wet weight of cell-floc or 0.30 g dry weight per flask). Approximately 25 to 33% of all cations assayed were adsorbed by the flocs present. Sulfate which is the predominant anion associated with the cations in mine drainage was also adsorbed to the extent of 25%. Iron appears to be the predominant cation present in the mine water (excluding H^{+}) when viewed on a mg/L. basis but this is not the case when it is considered on a molar basis. The following millimolar concentrations were calculated from the mine water control data in Table 18: Al (6.6), Ca (9), Fe (8.5), Mg (12), Mn (0.5), Si (1.4), NO_3 (0.3), and SO_4 (47.8). The total anion content

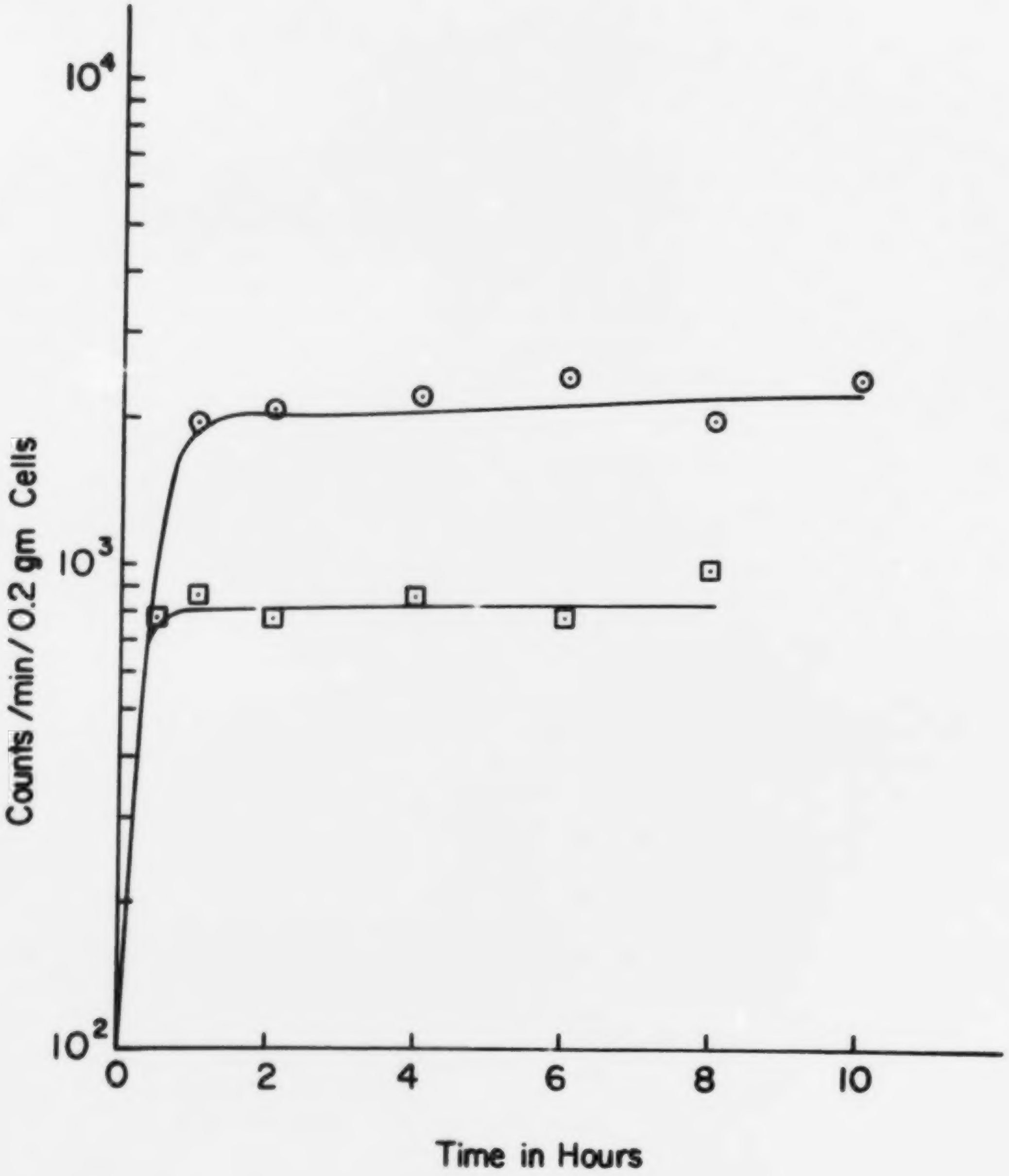


Figure 34. Accumulation of Zn^{65} plus 10 µg/ml carrier by a four-day culture of _Zoogloea ramigera_ 115 grown at 21 (⊡) and 28 (⊙)C

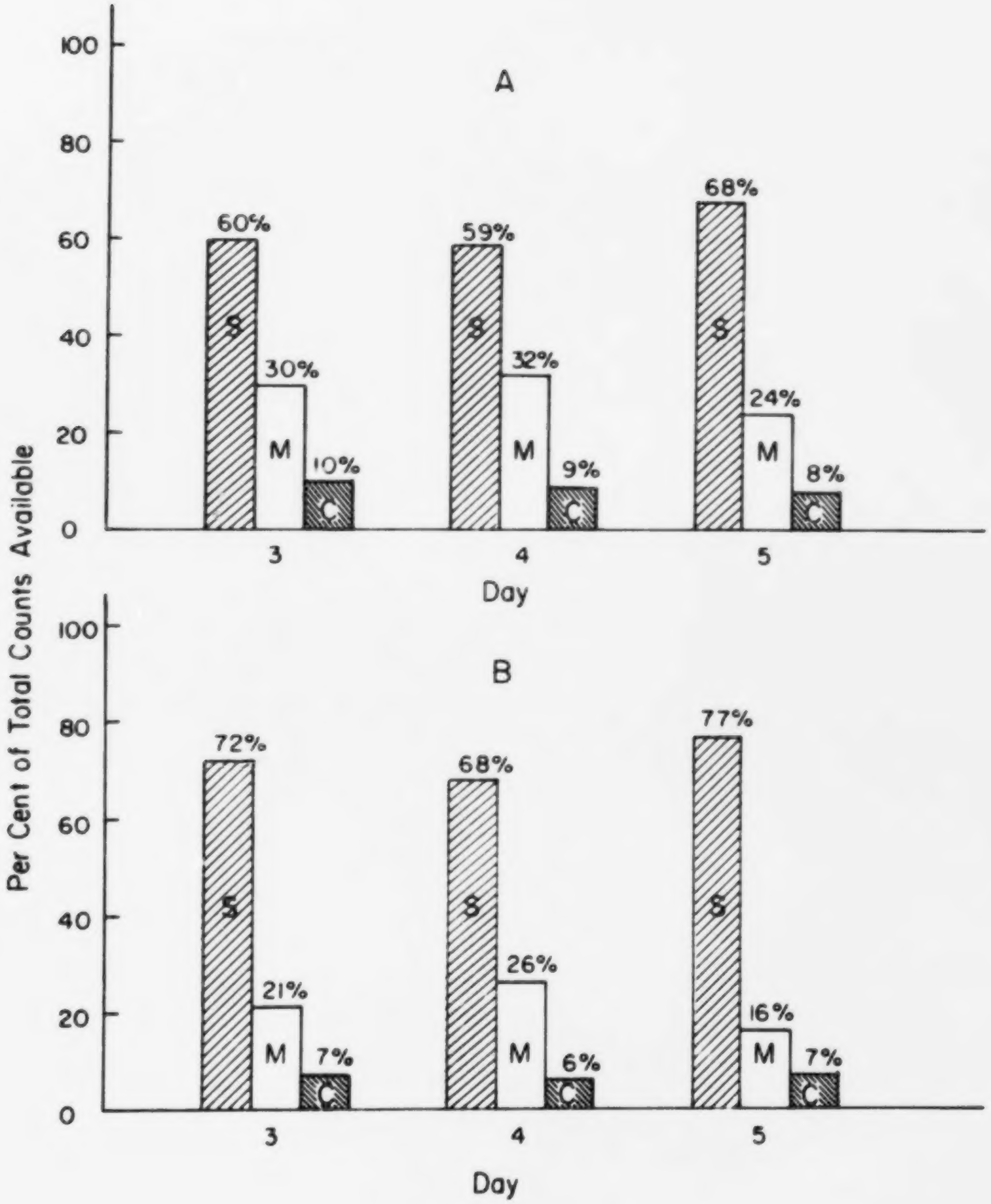


Figure 35. Interrelationship between the zoogloeal matrix and Zn^{65} absorption. (A) Addition of Zn^{65} at time of inoculation of culture of Zoogloea ramigera 115. (B) Addition of Zn^{65} after 3, 4, or 5 days incubation. S = supernatant, M = matrix, C = cells

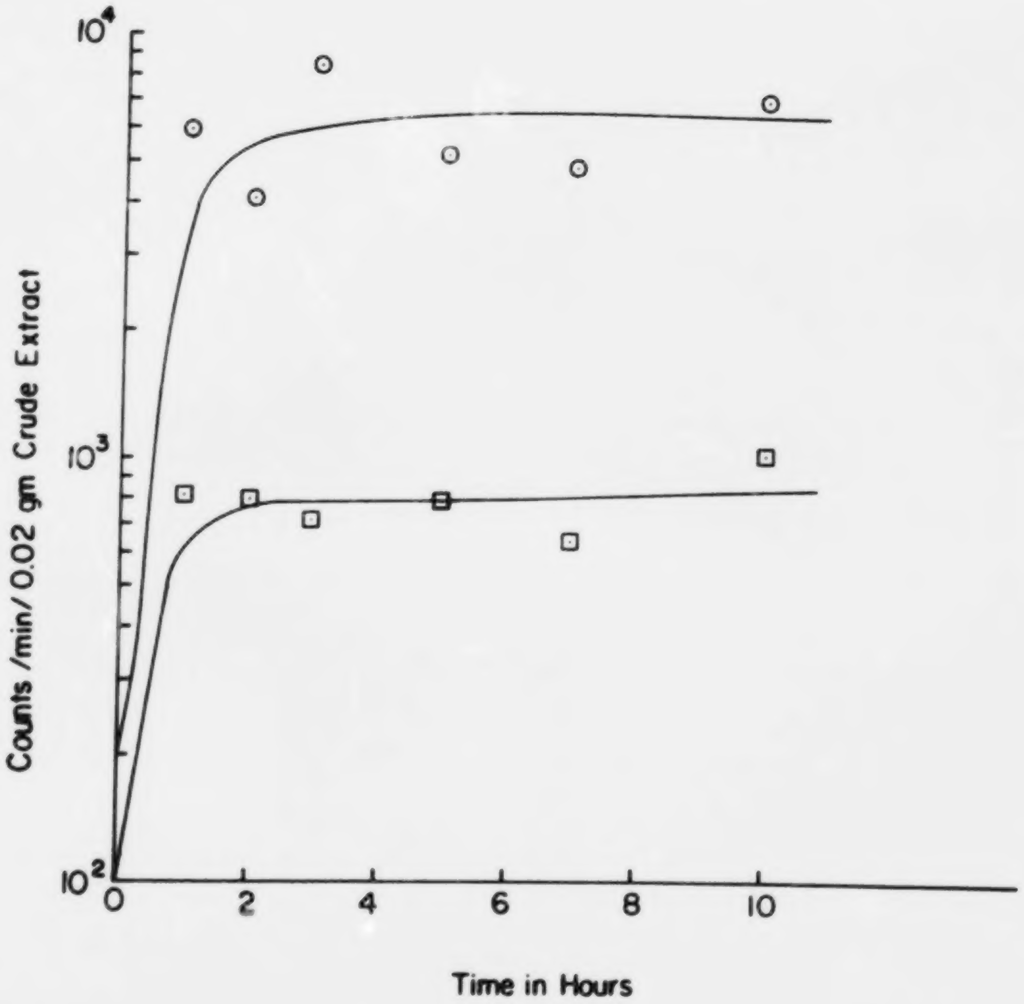


Figure 36. Uptake of Zn^{+2} by a crude extract of exocellular polymer of *Zoogloea ramigera* 115. Zn^{65} plus 100 μg/ml carrier (⊡); Zn^{65} plus 10 μg/ml carrier (⊙).

TABLE 18. Values (mg/L) showing difference in concentration of selected cations present in pH 3.0 acid mine water before and after contact with cell-floc

	Soluble Cation* (oxidation state not determined)						Soluble Anion	
	Al	Ca	Fe	Mg	Mn	Si	NO_3^-	$SO_4^=$
Control (Acid mine water)	180	360	475	280	30	33	18.2	4600
Sample (Avg. of 4)	120	285	342	202	22	28	16	3450
Change	-60	-75	-133	-78	-8	-10	-2	1150
Total removed from 50 ml	3mg	3.75mg	6.65mg	3.9mg	0.4mg	0.5mg	0.1mg	57.5mg
Millimoles removed	.11	.09	.12	.16	.007	.02	.001	.6

*Ag, Co, Cu, Cr, Li, Mo, Ni, Ti were all less than 2 mg/L in the mine water. They were determined but not included in the table.

detected was 48.1 millimolar and the total cation content was 38 millimolar. By difference the undetected cation is 10 millimolar and is presumed to be H^+. This corresponds to a theoretical pH of 2 as compared to the recorded pH 2.8 and probably reflects some undissociated HSO_4^-.

The total weight of ions removed by 300 mg cell-floc is calculated at about 76 mg. This represents an accumulation of 25% of its own weight by the cell-floc polymer.

It was assumed that the floc-polymer adsorbed cation and that the anion was adsorbed by association with the cation. The reverse was also a possibility and a series of experiments was carried out to examine the influence of anions on uptake of cations.

Results of these experiments are summarized in Table 19. The amount of Fe^{+3} as ($FeCl_3$) uptake by cell-floc was examined using two different concentrations of Fe (375 and 1160 mg/L. respectively, whereas the Cl^- ion content was 750 and 4000 mg/L. respectively). These analysis are designated as [A] in Table 19, and are compared to the uptake of $FeCl_3$ by cells which had been previously washed with one of the following salts: KH_2PO_4, KNO_3 or $MgSO_4$. Table 19 is divided into 3 sections: control or stock ion concentrations, sample solution values after 18 hours in presence of floc, and calculated difference values giving the concentration of ion removed (mg/L) from a 50 ml flask.

Wet weight of cell-floc in these experiments ranged between 32.36 g and 37.54 g per 50 ml flask. The PO_4, NO_3 and SO_4 values in the sample reflect the amount of PO_4, NO_3 or SO_4 taken up by cell-flocs during the washing procedure prior to addition of $FeCl_3$.

Comparison of [A] values in Table 19 indicates that virtually all of the iron was adsorbed by the floc when the initial concentration was 375 mg/L. That is, from 3.7 to 7.0 mg Fe remained in solution. When the initial Fe concentration was 1160 mg/L., 375 mg/l. remained after 18 hours indicating that the floc-polymer had reached its saturation point. However, when the cells were washed with KH_2PO_4 solution [B value] and then exposed to 1160 mg Fe/L., all of the Fe was adsorbed. This suggests that PO_4 is adsorbed in the cell-floc during washing and that Fe is associated with the PO_4. The PO_4 values verify this observation. PO_4 is taken up by floc and during the process more Cl^- is also adsorbed by the floc. That is the Cl^- difference is greater for the [B] values than for the [A] values. The same general observations hold for the NO_3 and SO_4 columns. No conclusions are drawn as regards SO_4 because it was added as $MgSO_4$. Mg was shown in Table 18 to be adsorbed.

Total weight of ions adsorbed to 300 mg dry weight floc in a 50 ml flask is calculated as 270 mg in the case of 1160 mg Fe/L., and indicates that the polymer has adsorbed nearly the equivalent of its own weight as $FeCl_3$.

TABLE 19. Values showing (A) concentration of Fe^{+++} and Cl^- ($FeCl_3$) absorbed by cell-floc, and by cell-floc after washing with (B) any one of three different anions

Ion Concentration (mg/L)

	Cell weight wet	Control					Sample after 18 hr					Difference				
		Fe	Cl	PO_4	NO_3	$SO_4^=$	Fe	Cl	PO_4	NO_3	SO_4	Fe	Cl	PO_4	NO_3	SO_4
A	32.26	375	750	-	-	-	7	408	-	-	-	368	342	-	-	-
B	32.26	375	750	900	-	-	0.5	125	670	-	-	374	625	230	-	-
A	34.46	1160	4000	-	-	-	373	**	-	-	-	787	-	-	-	-
B	34.46	1160	4000	900	-	-	1.2	292	593	-	-	1158	3708	307	-	-
A	34.87	375	750	-	-	-	4.5	342	-	-	-	370	408	-	-	-
B	34.87	375	750	-	1320	-	0.9	100	-	1065	-	374	650	-	255	-
A	32.78	375	750	-	-	-	8.7	433	-	-	-	364	317	-	-	-
B	32.78	375	750	-	-	480	4.7	92	-	-	390	370	658	-	-	90
A	37.54	375	750	-	-	-	3.7	370	-	-	-	370	380	-	-	-

*PO_4 added as KH_2PO_4, NO_3 as KNO_3, SO_4 as $MgSO_4 \cdot 7H_2O$

** Values not valid because of analytical interference.

Since it was apparent that total Fe uptake by floc increased with increasing concentration for a given time period, it was decided to examine this phenomenon in acidic mine water. For this purpose adsorption of Fe from mine water was compared to adsorption of Fe from mine water which contained added $FeCl_3$. Results of this experiment are tabulated in Table 20.

TABLE 20. Iron values (mg/L) showing adsorption of Fe from either pH 2.8 acid mine water or pH 2.8 mine water which was supplemented with $FeCl_3$

	Mine Water	Supplemented Mine Water
Weight of cell floc/flask	340 mg	260 mg
Fe control	370	600
Fe cell control	0	0
Fe sample (Avg. 3)	146	412
Fe difference	224	188
Total Fe adsorbed/50 ml flask	11.2 mg	9.4 mg
Total Fe adsorbed/g floc	33 mg	37 mg

Although the iron content was nearly doubled the amount adsorbed did not increase. The weight of cell-floc was less in the supplemented samples. However, when the weight of Fe adsorbed was calculated on a per gram of floc basis, the uptake was nearly equal. Unfortunately, a complete ion analysis for these samples was not made and differences in uptake of other mine water ions may exist.

The adsorption of ions by a culture of microorganisms that produce extracellular polymer has been demonstrated in Tables 18, 19 and 20. It is also known that production of polymer by cells can be stimulated via cultural manipulations. This would result in greater production of adsorption sites for ions if indeed the ions are adsorbed to the extracellular polymer.

Table 21 presents data showing the uptake of Fe and other mine water ions by polymer which was isolated and purified from the cultures previously described. The data in Table 21 illustrate that mine water ions are adsorbed by purified polymer. It must be pointed out that the purification processes alter the physical properties of the polymer and it may not be directly comparable to behavior in the culture situation.

Mine water cations are strongly adsorbed by the polysaccharide polymer synthesized by *Zoogloea ramigera* 115 cells. Association of anions with the adsorbed cations is apparent. The relative adsorption, approximating 25% for most ions, suggests a non-specific uptake. However, the relative affinities of ions for the polymer is not obvious from the available data and no conclusions are warranted with regard to selectivity.

TABLE 21. Values (mg/L) showing uptake of mine water ions by 50 mg purified polymer per 50 ml flask

	Fe	Ni	Co	Mn
Mine water	375	1.1	0.7	11.6
Polymer sample (Avg. 4)	297	0.5	0.35	5.5
Difference (uptake)	78	0.6	0.35	6.1
Total uptake per 50 ml	3.9 mg	.03 mg	.017 mg	0.3 mg

Ion adsorption to polymer promotes flocculation and affords a means of physically separating the ions in a highly concentrated form from mine water. In spite of lack of cation specificity, significantly high amounts of iron and sulfate ions were removed from solution to attract consideration of the process for pollution abatement purposes in selected situations. Such a process could become very attractive if ions having a market value were present in the solution. Production of cell-flocs is a potentially low cost process and increased polymer synthesis by cells is a possibility which could increase the ion adsorptive capacity of the cultures.

Many chlorinated hydrocarbon insecticides have been isolated from surface waters, usually in concentrations of less than 1 μg/liter or 1 part per billion (ppb). Significance of pesticide in water has been established (25). Our interest is in the fate of these chemicals in a water column and particularly in their adsorption to silt and floc forming bacteria which form contemporary sediment in lakes. Bacterial floc is an aggregation of cells which results in a macroscopic bacterial clump that settles from liquid leaving that medium less turbid. This type of growth appears to result from physical, chemical, and biological interactions when extracellular fibrillar polymers are synthesized by organisms (13, 43).

A preliminary study of aerobic bacteria isolated from Lake Erie indicated that of 33 isolates tested in 6 different growth media, 19 formed flocs in at least one medium, whereas 10 formed flocs in two or more of the media. Two of the floc forming isolates were selected for further study with regard to their ability to concentrate and accumulate the pesticide aldrin from solution. One bacterium was an orange-red pigmented gram negative (G-) rod, tentatively identified as either a Flavobacterium or Protaminobacter. The other was a gram positive (G+) Bacillus species.

Our experimental procedure was as follows: The test organisms were grown in shake culture at ambient temperature (22° ± 2°C) in nutrient broth (8 g/L., Difco), harvested by centrifugation, washed 2x with distilled water and resuspended in 25 ml of distilled water. Erlenmeyer flasks containing 50 ml suspensions of bacterial floc were then placed on a rotary shaker and 1 ml of aldrin dissolved in acetone was added to give a final concentration of 1×10^{-6} g aldrin/ml or 1 part per million (ppm).

After being shaken at 120 rpm for the desired time period, the flasks were removed from the shaker and the floc was separated from the supernatant by centrifugation. The flocs were washed twice with distilled water and the washings were added to the original supernatant. The pesticide exposure time was calculated as that period between addition of aldrin to the solution and the separation of the second washing from the bacterial floc. The floc and supernatant fractions were extracted separately with a 3:1 mixture of heptane and acetone. The organic solvent fractions containing the aldrin were concentrated by evaporation and adjusted to a volume of 4 ml. Two μl of samples were injected into an Aerograph model 200 gas chromatograph (GLC) utilizing an electron capture detector.

Total aldrin adsorbed to bacterial floc vs. time is plotted in Fig. 37. The theoretical maximum for adsorption calculated from a standard curve was 1 ppm aldrin. Recovery values for aldrin varied with individual experiments between 70 and 130% (0.7 to 1.3 ppm), possibly due either to adsorption on glassware (7), or to varying sensitivity of the electron capture detector. Almost all of the aldrin adsorption to floc took place within the first 20 minutes of contact. The amount of adsorption in most cases remained nearly constant after 20 min. This was verified statistically by single variable linear regression at both the 1 and 5% levels of significance when aldrin adsorbed vs. time was compared. All of the aldrin added to the G^+ bacteria was recovered from the floc; none was recovered from the supernatant. The recovery value averaged 88%. The slope of the line is linear and $\beta = 0$ at both the 1 and 5% significance levels when floc weight vs. pesticide adsorbed was evaluated. This is due to the high rate of aldrin adsorption within the minimum time period required to obtain the first adsorption value (i.e. 12-15 min.). Lack of significant difference in adsorption by 0.041 g floc compared to 0.087 g floc indicates that 0.041 g was sufficient to adsorb all of the available aldrin.

Adsorption curves shown in Fig. 37 indicate a rapid uptake of aldrin for the first 20 min until maximum theoretical adsorption is reached. As aldrin is adsorbed, less is available in solution to be adsorbed. Therefore, the flocs effectively adsorb from more dilute solutions than anticipated by the initial test concentration. This would essentially represent adsorption from lower more realistic pesticide concentrations found in Lake Erie.

Data for the G^- organism show that the amount of aldrin adsorbed by an equal weight of cell flocs remained the same or increased only slightly with time beyond 20 minutes and either decreased or remained unchanged in the supernatant. The adsorption curve was linear at the 1 and 5% significance levels, but only if the 0.0656 g. wt. was omitted from the calculations. If included, the data was not linear at either level. However, in neither case did $\beta = 0$, indicating a relationship between adsorption and floc weight in this case. This results from a slower adsorption by the G^- bacteria as compared to the G^+.

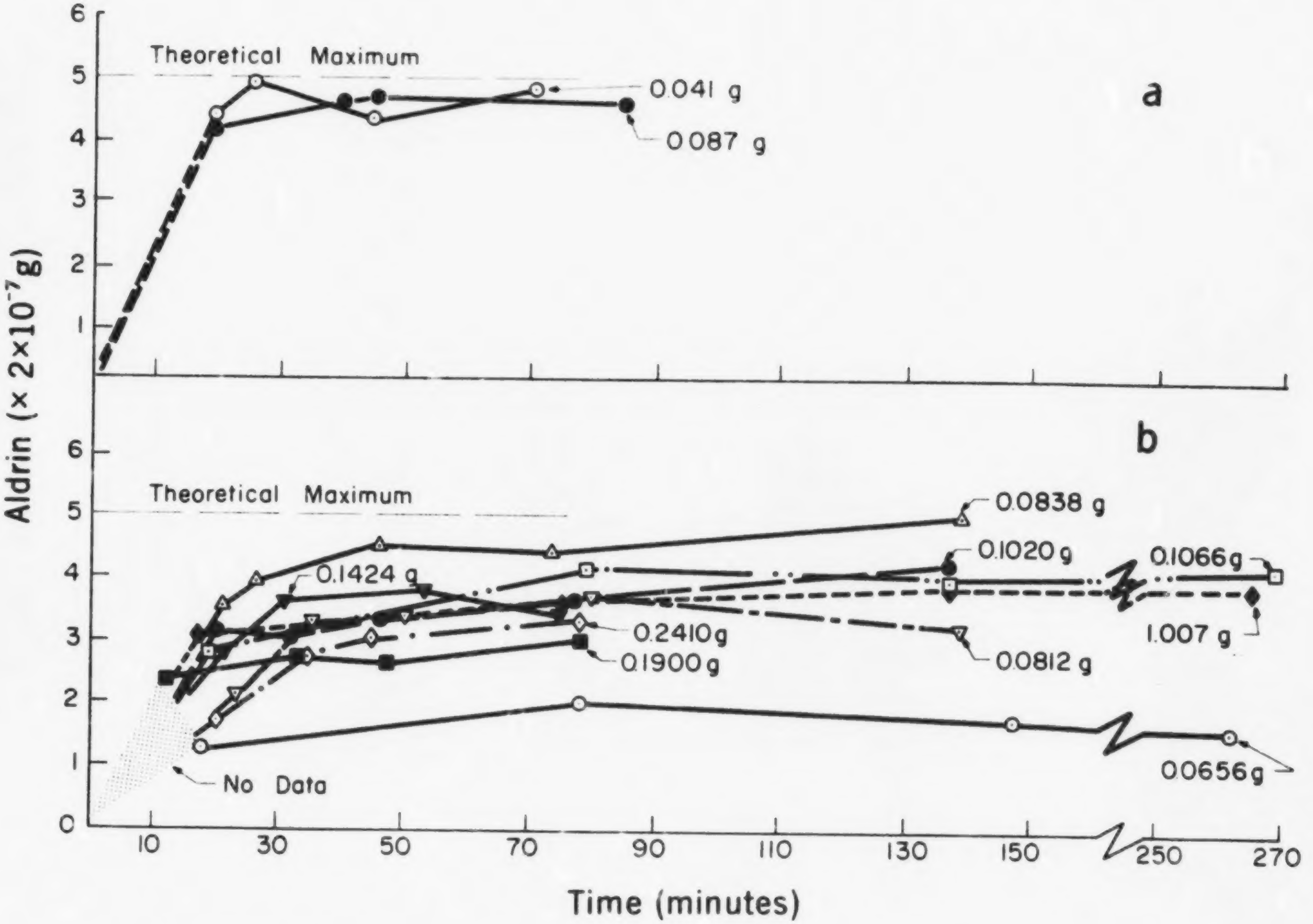


Figure 37. Curves showing adsorption of aldrin by both (a) gram positive bacterial floc and (b) gram negative bacterial floc vs time. Numerals with each curve indicate the dry weight of bacterial floc used in each experiment. Broken or dotted lines indicate extrapolation from the first experimental point to 0 time

The concentrating effect of these bacteria is considerable. For example, when 0.041 g of G^+ floc adsorbed pesticide from 25 g water, it represented a concentration factor of about 625 to 1 within 20 minutes. A similar but slightly smaller amount of adsorption occurred with the G^- organism. Analogous findings have been reported for algae (57, 95).

Samples of natural sediment which were in the process of settling and accumulating in Lake Erie were collected by specially designed sediment collectors placed on reefs (49). This sediment consisted primarily of inorganic matter. In addition to being analyzed in a manner identical to that described for bacterial floc, it was also examined on a Dohrmann Instrument Co. model 200A microcoulometer.

Aldrin and dieldrin were detected in contemporary sediment by both GLC and the microcoulometer. Additional aldrin added experimentally was adsorbed and as shown in Fig. 38, after 10 and 70 minutes the concentration was almost equal. No aldrin was detected in the supernatant. The data were linear at both the 1 and 5% levels of significance and $\beta = 0$.

Contemporary lake sediments appear to accumulate pesticide from suspension in a manner similar to that shown for bacterial floc. Floc-forming bacteria are common in the lake environment and experimentally have a rapid and high adsorption capacity for aldrin. Organic "floc-like" bottom sediments from the eastern basin of Lake Erie have been reported (89) and electron microscopic examination of contemporary sediments from Lake Erie shows that it is a conglomerate of bacteria, diatoms, inorganic and detrital particulates. Counts of 10^5 aerobic and 10^6 anaerobic heterotrophic bacteria have been obtained per gram wet weight of contemporary lake sediment. In this regard, clay particles are known to adsorb pesticide and lindane is known to adsorb to lake sediments (60). Pfister et al. (77) reported that chlorinated hydrocarbons both behave as suspended microparticulates and are associated with other microparticulates including detritus in the water-column. Others (71) have established that DDT was taken up from organic detritus by fiddler crabs. Significant concentrations of chlorinated pesticides have been detected in algae and lake bottom mud (55). It is known that actinomycetes, fungi and other bacteria adsorb and concentrate pesticides from solution (20, 97) and that microparticulates associate with microorganisms (76). Particulate organic material has been considered a potentially important source of food for filter feeding marine organisms (3). The suggestion has been made that most of the particulate organic carbon at depths shallower than 175 m in the Atlantic Ocean off South America consisted of living organisms and decomposable organic matter (52).

It is concluded that floc-forming microorganisms act as adsorbants for other suspended microparticles including chlorinated hydrocarbons and this represents a natural process for removal of microparticles from the water-column.

Once settled from suspension the fate of pesticides is in question, but they may be degraded under anaerobic conditions (51). It is likely that

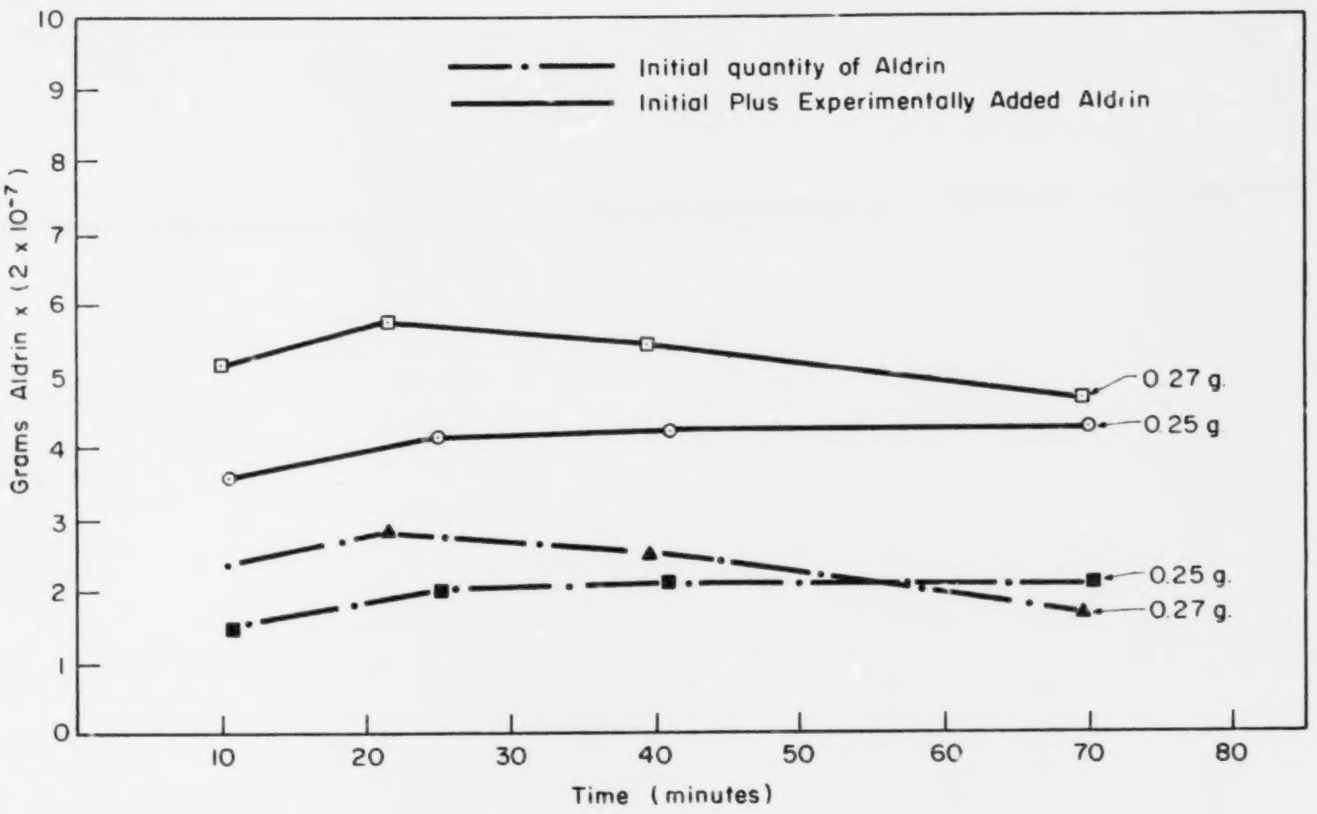


Figure 38. Curves showing amount of aldrin found in two different contemporary sediment (silt) samples, and the additional aldrin adsorbed experimentally by the two samples vs time

pesticides concentrated in bottom sediments for even short periods of time would exert an insecticidal effect on the bottom insects and other susceptible fauna. Jensen and Gaufin (54) and Carlson (18) have shown that different species of stonefly and mayfly naiads have varying susceptibilities to the same pesticide. This may explain the disappearance of certain insects from Lake Erie, such as mayflies, and the persistance or increase of others. The same may hold true for other organisms in the lake.

Electron microscopic examination of contemporary sediments from Lake Erie shows that it is a conglomerate of bacteria, diatoms, inorganic and detrital particulates. Figures 39a and 39b are photographs of a scanning electron micrographs showing contemporary sediment. Diatoms and detrital material are clearly visible.

Polymer Production

Zoogloea ramigera 115 in liquid, shaken culture becomes extremely viscous and then semi-solid in the presence of 0.5 to 2.0 glucose, so that the flask can be inverted without disturbing the culture. This is referred to as total thickening or gelled culture. Figure 40 illustrates the extent of polymer synthesis when Z. ramigera was cultivated in a 250 ml Erlenmeyer flask containing Crabtree's arginine - salts medium supplemented with 2% glucose. A plug of culture has been removed to aid in visualizing the consistancy of the material. Rate of total culture thickening was observed to be dependent upon glucose concentration in the culture medium. Cells cultured in basal medium which contained fructose, galactose, glucose, lactose, mannose, soluble starch, or sucrose as the supplemental carbon source, all produced abundant and nearly equivalent amounts of polymer. Since the polymer synthesis increases in response to the concentration of carbon source in the medium it would be reasonable to assume that the organism would produce small amounts of polymer in low organic environments. It could then be surmised that the organism would be more prevalent in the aquatic habitat than previously thought, but might not be recognized as Zoogloea because of lack of capsular material synthesis in the absence of high organic carbon nutrient concentrations.

Paper chromatograms of acid-hydrolysed polymers isolated individually from cultures grown in various carbohydrates, although revealing only one carbohydrate spot when developed in isopropanol:water or in butanol: acetic acid:water as reported by Friedman et al. (42), revealed two distinctly separate spots for glucose (Rg. 1.00) and galactose (Rg 0.87) when developed in an ethyl acetate;pyridine;water system. These correspond with Rg values of known glucose and galactose in the same system. It was concluded that the polymer was a polysaccharide composed of glucose and galactose with glucose as the predominant sugar. Cells grown in a basal medium plus fructose, galactose, glucose, lactose, mannose, soluble starch, or sucrose all produced polymer consisting of only glucose and galactose.

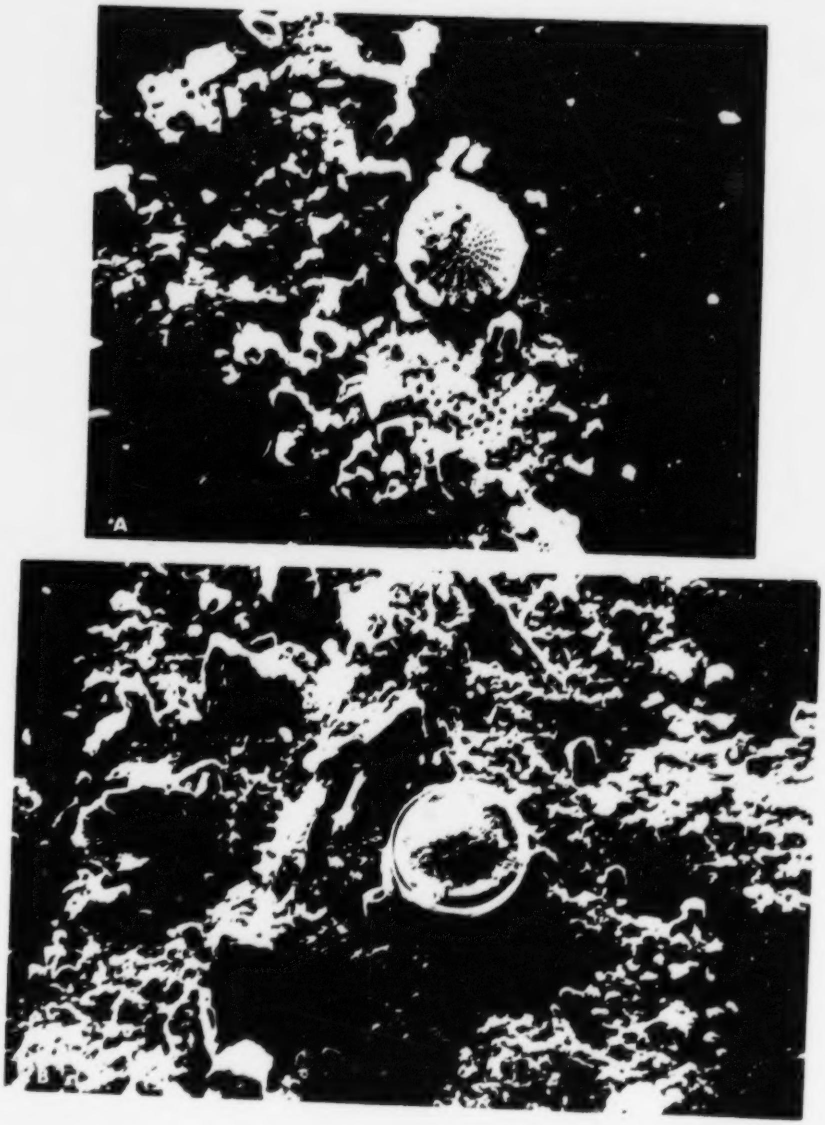


Figure 39. A and B are electron scanning micrographs of contemporary sediment from Lake Erie Showing diatoms, detritus and particulate matter X5500

Figure 40. Photograph of a 50 ml culture of Z. ramigera 115 in a 250 ml Erlenmeyer flask laying on its side illustrating the semi-solid consistency of the culture due to polymer synthesis. A core plug has been removed from the culture to aid in visualizing the material.

Since the carbohydrate source did not affect the composition of the extracellular polymer, glucose was selected as the carbohydrate source for all further investigations. The time sequence of polymer productions was investigated in a 14 L. fermentor apparatus. The cells were so firmly enmeshed in the matrix polymer that it was not possible to obtain cell counts. Therefore cell growth was followed by a total DNA determination using a diphenylamine assay. Utilization of glucose available in the medium was followed by a combination of the anthrone test for total carbohydrate and Nelson's test for reducing sugar.

Figure 41 represents the results of one study obtained from a 10 L. fermentor culture over a period of ten days. Utilization of glucose available in the medium is related to viscosity of both supernatant and whole culture aliquot and to accumulation of PHB. The level of glucose dropped in 58 hours from 62 micromoles to 4.0 micromoles and was therefore not available in the culture solution between 60 and 240 hours when the viscosity was observed to increase. PHB concentration rapidly increased between 20 and 40 hours and reached a maximum between 60 and 70 hours suggesting that PHB formation stopped when the glucose was spent. After 75 hours there was a steady decrease in the quantity of PHB. At approximately 60 hours, viscosity of both supernatant and whole culture showed

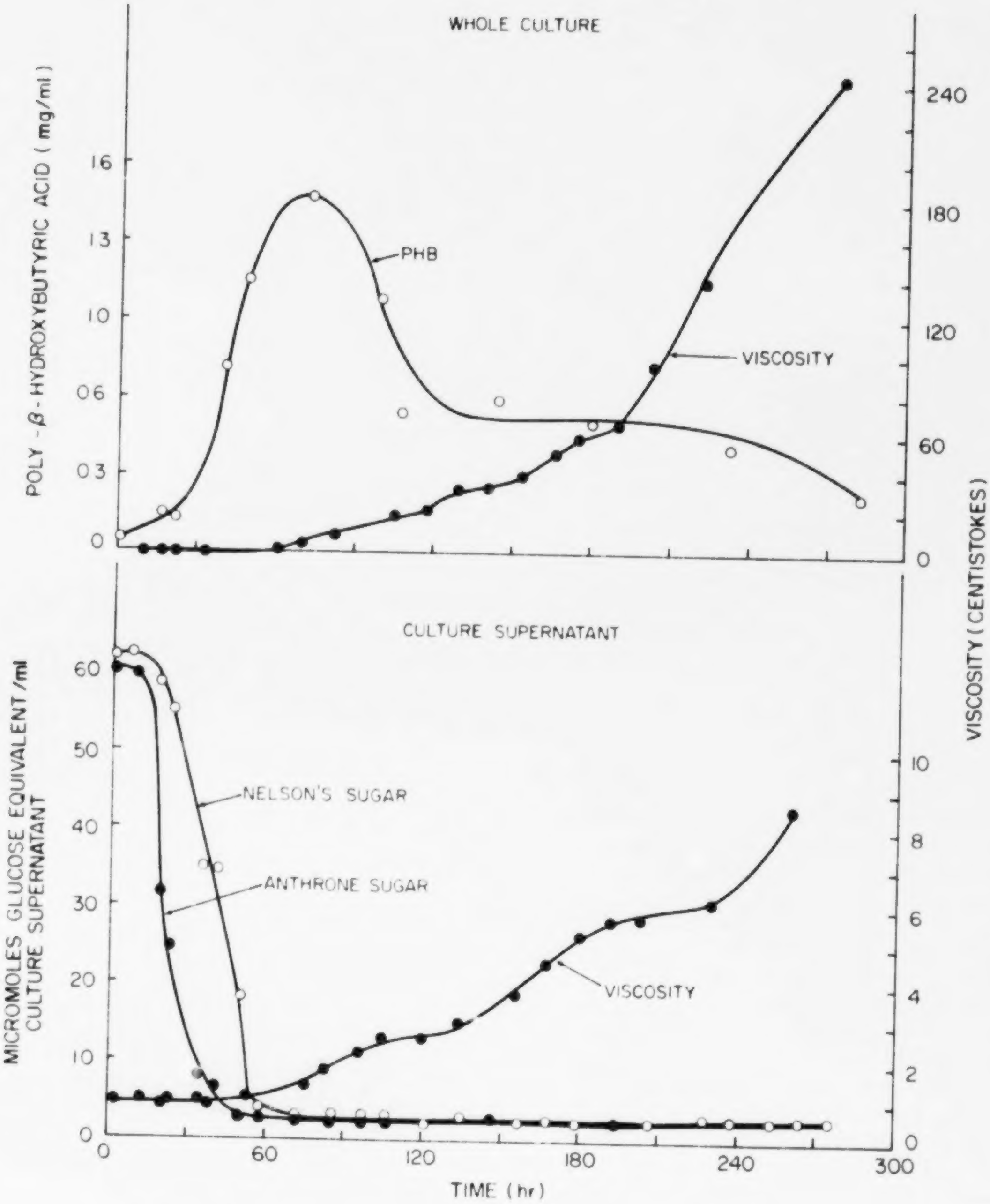


Figure 41. Curves showing viscosity of both whole culture and supernatant, sugar available in medium measured by both Nelson's and anthrone tests, and PHB accumulation in Z. ramigera 115 culture vs time. Growth in 10 L batch fermenter at 24° ± 2°C

a marked rate of increase suggesting a relationship between PHB expenditure and polymer synthesis. At 180 hours an accelerated rate of increase in viscosity was observed. In the course of the study, the viscosity of the supernatant increased from 1.00 to 9.5 centistokes. In the same time period the viscosity of the whole culture aliquot increased from 1.0 to 680.0 centistokes. At this point, it became extremely difficult to centrifuge enough supernatant from the samples to allow viscosity measurement of the supernatant.

The rapid disappearance of glucose from the medium as determined by both anthrone and Nelson's tests could be due either to rapid metabolism of glucose or to physical adsorption of the sugar by this organism. Z. ramigera isolate 115 has been shown to adsorb and accumulate metal ions and organic matter in its matrix and the glucose could be adsorbed to the matrix polymer (41). Crabtree (1966, Ph.D. Dissertation, Madison, Wisconsin) studying a different isolate (I-16-M), has previously associated the rapid initial uptake of glucose with the accumulation of PHB. In the present study PHB accumulated within the cell, then as the viscosity of the supernatant and of the whole culture increased, the quantity of the PHB decreased. Perhaps PHB is an intermediate storage compound that is utilized as a metabolite for extracellular polymer production or for synthesis of active precursors.

Figure 42 represents the results of a similar study on another fermentor culture over a period of twenty days. The DNA assay data approximates the familiar bacterial growth curve. As the total amount of DNA reached a maximum at 96 hours, over half of the available sugar had disappeared from the medium. After 250 hours the reducing sugar present in the medium leveled off at 0.08 micromoles/ml or less. The total carbohydrate, as reflected by the anthrone test data, leveled off at 11 micromoles in the same time.

In this study the viscosity of the culture supernatant increased from 1.0 to 10.3 centistokes. The viscosity of the whole culture aliquot, which contained cells and polymer increased to 130 centistokes. In other batch cultures the whole culture viscosity has been measured as high as 1055 centistokes before total thickening made viscosity measurement physically impossible.

Figure 43 illustrates the disappearance of starch from the growth medium when it was added in place of sugar as the carbohydrate source. Curve A shows that all starch was hydrolysed within 10 hours. Total carbohydrate assayed by the Anthrone procedure decreased less rapidly than starch indicating either that starch was hydrolyzed more rapidly than glucose was metabolized or that glucose was converted to new polysaccharide. The latter is a likely possibility because total Anthrone positive carbohydrate did not go below 20 micromoles per ml; whereas reference to Fig. 42 shows that Nelson's reducing sugar (in this case, glucose) was completely utilized. One other explanation for rapid starch loss would be production of Scardinger dextrins (5 to 7 glucose units) that were not yet metabolized but would not react with iodine as starch.

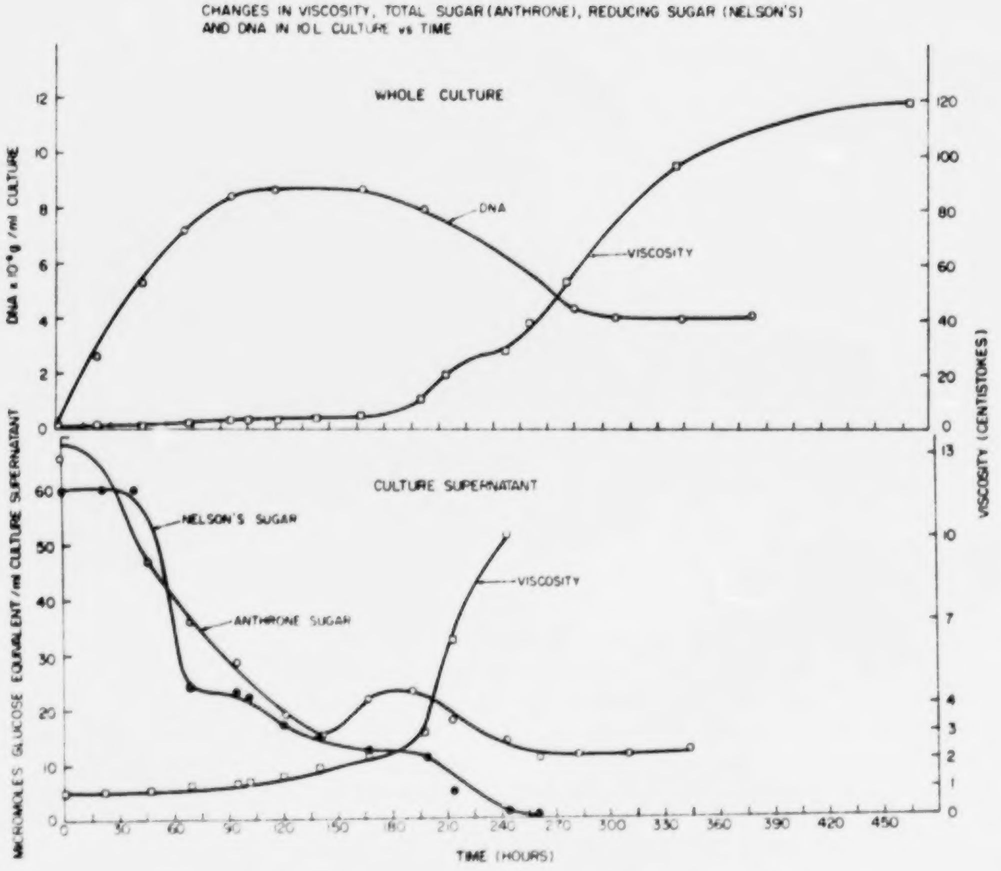


Figure 42. Curves showing viscosity of both whole culture and supernatant, sugar available in medium measured by both Nelson's and anthrone test, and total DNA in Z. ramigera 115 culture vs time. Growth study in 10 L batch fermenter at 24° ± 2°C

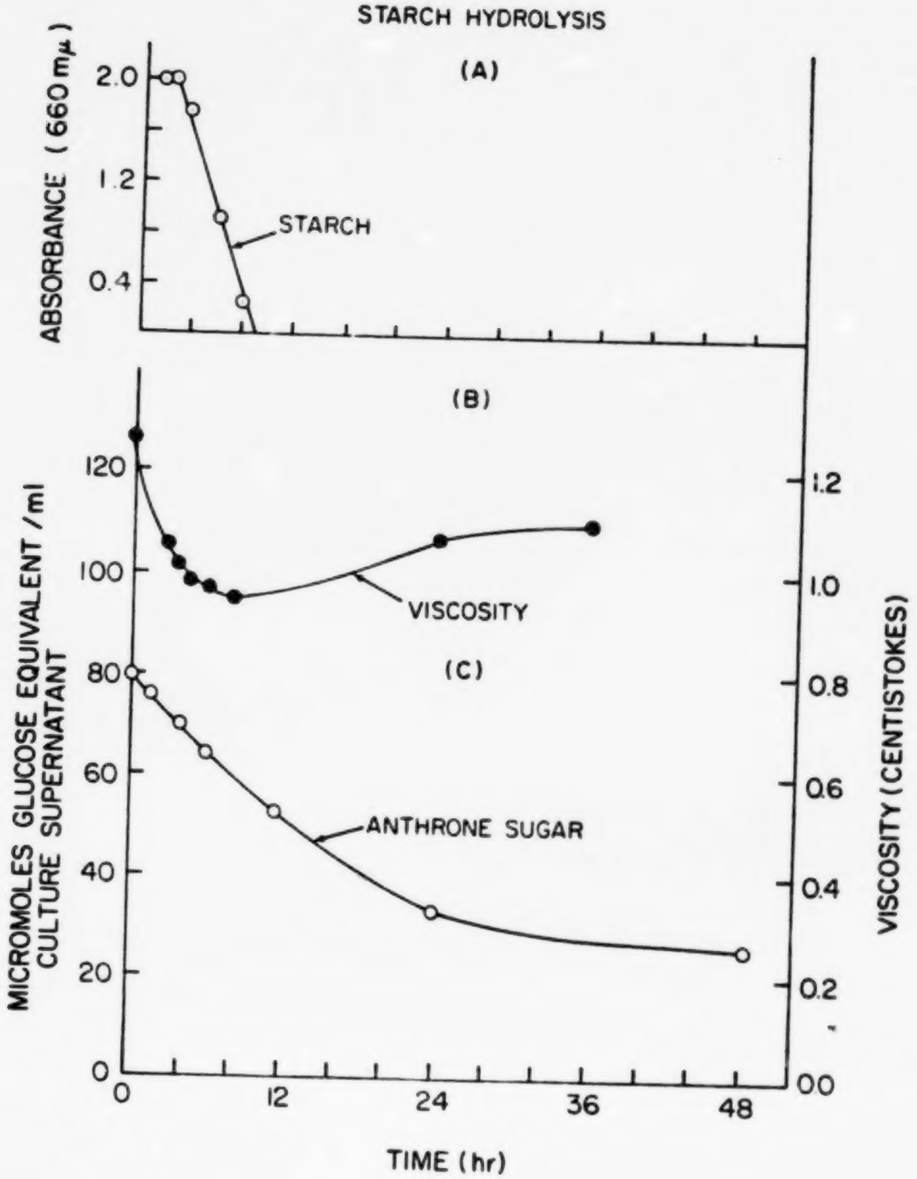


Figure 43. Curves showing starch hydrolysis, change in viscosity, and loss of total carbohydrate (Anthrone) in *Z. ramigera* isolate 115 culture vs time

The above observations have been made on 17 different fermentor runs and the following interpretations seem reasonable. After the cells had ceased to multiply as suggested by the leveling off of the DNA, viscosity of both supernatant and whole culture continued to increase. This continued increase in viscosity could be due to increasing molecular weight, increasing branching, modification of the polymer such as a change in water binding properties, or further synthesis of more short chain polymer. Figure 41 shows PHB still decreasing after 180 hours. The steadily increasing viscosity in the supernatants suggest the formation of a polymer precursor or low molecular weight polymer which remains in solution at 6600 x g. The molecular weight of the polymer probably increases until it reverts from a soluble molecule to a suspended molecule. Continued polymerization results in a matrix which flocculates from suspension and ultimately forms a gel. Viscosity in this situation can best be measured in whole culture aliquots to reflect increase due to both suspended and dissolved polymer.

The efficiency of glucose and arginine conversion to polysaccharide has been estimated from the data derived from several different fermentor studies. Cellular DNA was assumed to be 3% of the dry weight of the cells. Comparison to DNA-growth curves indicated a production of 4.6 g cells/L. at the point of total culture thickening. This value was compared to the total cell-floc dry weight value of 8.065 g/L which was harvested from the culture vessel by centrifugation at the same time. The calculations indicate that the cells comprise about 55% of the total cell-floc dry weight. Total ash values in these experiments accounted for less than 6% of the dry weight and it was assumed therefore that the extracellular polymer accounted for about 40% of the weight. If it is assumed that 50% of the cell dry weight and 40% of the extracellular polymer is carbon we can account for 3.83 g carbon/L of a theoretical 4.22 g carbon/L added as glucose plus arginine. The conversion of carbon substrate to cells is therefore estimated to be 54% and the conversion to polymer is approximately 34%. It has been previously reported that the floc-polymer adsorbs 99 x its own weight of water (32). That is, the ratio of wet weight of culture gel to dry weight is 99:1.

Zoogloea ramigera 115 has the capacity to produce prolific quantities of extracellular polymer that has the capacity to adsorb metal ions and/or organics from solution, to effect the removal of these materials from water by flocculation and settling processes. Increased knowledge of polymer production could lead to a workable system for industrial pollution abatement.

SECTION VI

GENERAL DISCUSSION OF THE IMPLICATIONS OF MICROBIAL POLYMER SYNTHESIS IN WASTE TREATMENT AND LAKE EUTROPHICATION

The high metabolic rates found in most bacteria establish them in a predominantly significant role in treatment processes.

Aerobic waste treatment plants are designed to take advantage of these biological events. If the treatment system is viewed as a continuous culture ecosystem comprised of a mixed microbial population, it can be asserted in general terms that the biomass density is directly related to nutrient concentration or sewage strength and the rate of BOD removal (cell growth) is directly related to rate of flow through the treatment plant.

As an ecological consideration there is no reason to consider the conversion of oxidizable pollutants entering on oligotrophic lake or the ocean (at the lowest trophic level) as anything different, except that the overall system is more dilute and therefore the flocculent biomass concentration will be less. In this regard, a highly eutrophic lake such as Lake Erie, can be considered somewhere between an oligotrophic lake and an aerobic treatment system. Indeed we find an intermediate amount of flocculent sludge accumulation in Lake Erie. We are not so crass as to imply that consideration of a lake system is not more complicated than the above generalities may suggest - particularly at higher trophic levels. However, at the lowest trophic level we can consider the processes described as a by-pass around primary productivity which contributes to the initial biomass formation both directly and by stimulating primary productivity.

Relationship of Extracellular Polymer to Flocculation

We have been studying a group of bacteria that have been historically recognized as primary contributors to aerobic waste treatment (1, 14, 15) with particular interest in the organisms' ability to flocculate and to concentrate substances from solution. Several floc-forming bacteria have recently been isolated from waste water in our laboratory and elsewhere by other investigators. The isolates from aerobic treatment systems are characteristically gram negative pseudomonad type rods, some of which produce an easily recognized zoogloeal or capsular matrix and are given the generic name Zoogloea.

All of the floc-forming bacteria examined in this laboratory, with the aid of electron microscopy, have been observed to produce extracellular polymer fibrils. The polymer fibrils around the cells may be manifest as a zoogloeal matrix, capsule, slime, or non-observable by bright field microscopic techniques. Figure 27 shows the extracellular fibrils which surround the cells that are characteristic of the type of flocculent bacterium which appear as suspended particles but which do not produce the typical zoogloeal matrix. Figure 17 was prepared using a freeze etching technique and shows the fibrillar strands that comprise the zoogloeal matrix around *Z. ramigera* 115. Several cells are embedded within the fibrillar network of the matrix, and an interface that delineates the matrix from the surrounding menstruum can be seen. Details concerning matrix formation as well as fine structure and chemical composition of the extracellular polymer have been published elsewhere.

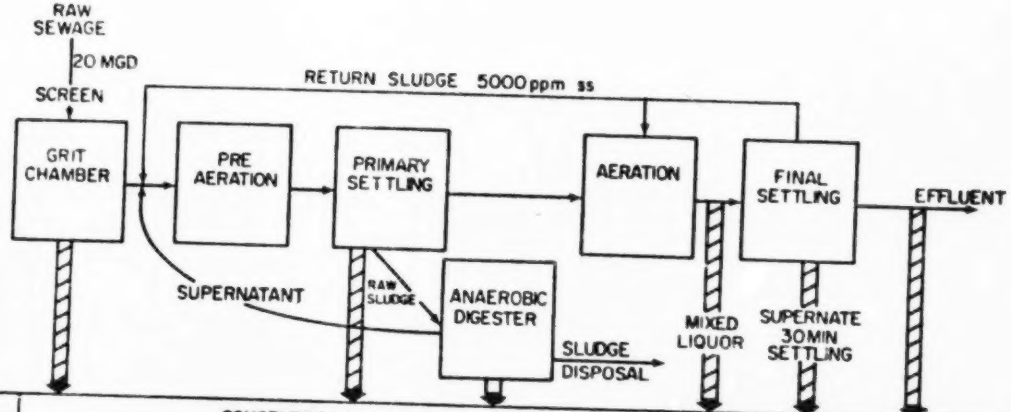
It has been postulated that the extracellular fibrils are responsible for the flocculent growth habit of the organisms by entanglement of cells among fibrils or by adsorption of cells to fibrils. The physical and chemical properties of fibrils around different species of bacteria will determine the extent to which water is bound to the polymer and will also determine solubility properties of the polymer and affinity for adsorption of dissolved chemicals.

Chemical Adsorption by Extracellular Polymer

The zoogloeal matrix of isolate 115 as well as the floc of *Z. ramigera* isolate I-16-M have been shown to possess a high affinity for transition metal cations. For example, flocs of *Z. ramigera* 115 adsorbed the following ions from solution and increased the dry weight of the floc by the percentage shown: 34% of the weight as Cu^{+2}, 25% as Co^{+2}, 17% as Fe^{+3}, and 12.5% as Ni^{+2}.

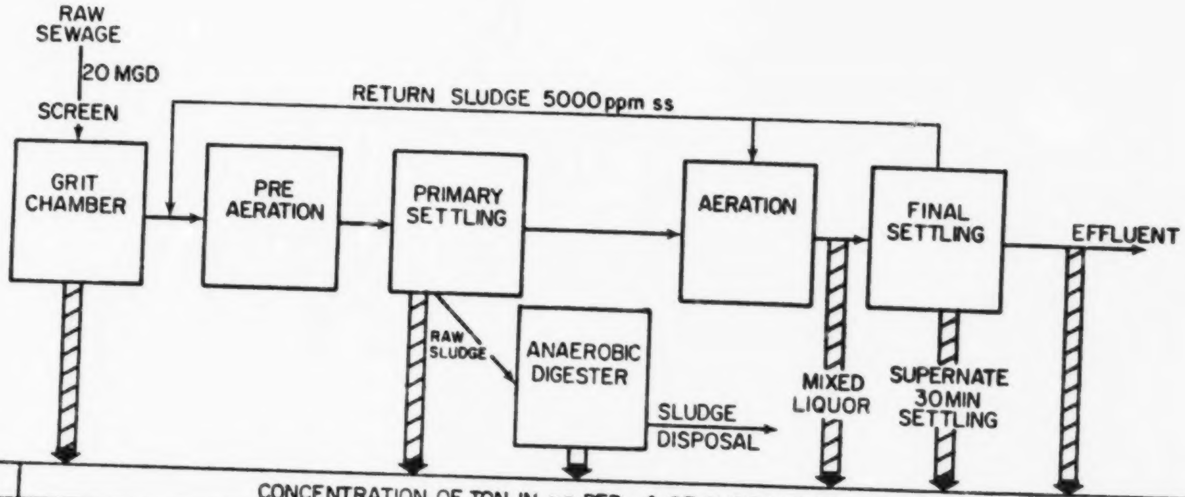
The above data were obtained on flocs in which cells were embedded. Subsequent studies on purified matrix polymer isolated from *Z. ramigera* 115 established that the polymer behaves as a polyelectrolyte and also adsorbs metal ions from solution. A collodial suspension of purified polymer in the presence of certain metal ions will flocculate as a polymer-metal ion complex and separate from solution or suspension. It appears therefore, that the polymer fibrils which make up the characteristic zoogloeal matrix are responsible for the unique adsorptive capacity of the floc.

Relationship of ion adsorption to waste treatment and eutrophication. The above observations can be used to explain some of the metal ion relationships observed in activated sludge treatment systems. For example, the schematic flow diagram of a municipal treatment plant shown in Fig. 44 shows the concentrations of some metal ions at various stages of the process. The cation concentration of the supernatant after primary settleing was generally less than that of raw sewage. The difference reflects the amount of ion which was adsorbed by the settled sludge-floc. Since adsorption from the bulk solution to floc represents a concentration from a large volume to a small volume, the ion concentration in the anaerobic digester was increased significantly. The concentration of ions in the digester supernatant was 20 to 100 times greater than in the raw sewage. This is recycled, giving a net buildup of ions in the mixed liquor when compared to raw sewage. Figure 45 represents data obtained at a later date from the same system shown in Fig. 44, after the practice of returning digester supernatant had been stopped. However, the data pertaining


RAW SEWAGE
20 MGD
SCREEN
GRIT CHAMBER
PRE AERATION
PRIMARY SETTLING
RETURN SLUDGE 5000 ppm ss
AERATION
FINAL SETTLING
EFFLUENT
SUPERNATANT
RAW SLUDGE
ANAEROBIC DIGESTER
SLUDGE DISPOSAL
MIXED LIQUOR
SUPERNATE 30 MIN SETTLING

METAL ION	CONCENTRATION OF ION IN μg PER mℓ OF SUPERNATANT FROM LOCATIONS INDICATED					
Cd	0.01	0.01				
Cr	0.54	0.17	32.32	1.69		
Cu	0.12	0.09	11.13	0.49		
Fe	1.52	1.18				
Mn	0.19	0.19				
Ni	0.19	0.12	4.06	0.37		
Zn	0.42	0.25	9.49	1.82		

Figure 44. Flow diagram of a treatment plant (Canton, O.) showing concentration of metal ions at various locations


RAW SEWAGE
20 MGD
SCREEN
GRIT CHAMBER
PRE AERATION
PRIMARY SETTLING
RETURN SLUDGE 5000 ppm ss
AERATION
FINAL SETTLING
EFFLUENT
RAW SLUDGE
ANAEROBIC DIGESTER
SLUDGE DISPOSAL
MIXED LIQUOR
SUPERNATE 30 MIN SETTLING

METAL ION	CONCENTRATION OF ION IN μg PER mℓ OF SUPERNATANT FROM LOCATIONS INDICATED			
Cd	0.04	0.02	0.17	0.01
Cr	0.13	0.13	1.74	0.07
Cu	0.04	0.06	0.86	0.02
Fe	1.06	0.55	9.75	0.22
Mn	0.22	0.20	1.29	0.12
Ni	0.28	0.21	0.99	0.23
Zn	0.48	0.30	2.86	0.05
Pb	0.28	0.19	0.79	0.21

Figure 45. Flow diagram of a treatment plant (Canton, O.) showing concentration of metal ions at various locations

to mixed liquor and supernatant of settled mixed liquor indicates that the metal ions were adsorbed to floc, and this sludge was recycled to the system. The data presented in Figs. 44 and 45 are actual plant data and are not experimental which unfortunately precludes a comparison of values at each location throughout the system.

Organic adsorption to polymer and relationship to waste treatment and eutrophication. Laboratory investigations of the floc-polymer of *Z. ramigera* isolates 1 and 115 show that the flocs actively metabolize a wide variety of amino acids, proteins, urea, and NH_4^+ as nitrogen sources. Both isolates could hydrolyze casein, gelatin, starch and urea and isolate 115 could hydrolyze collagen. The cells can use a variety of organic acids, alcohols, sugars and sugar alcohols as carbon sources. This suggests that proteinaceous and other natural organic wastes are hydrolyzed by these floc-formers and any liberated amino acids would be converted by amino acid oxidizing enzymes to corresponding organic acids plus NH_4^+. The organic acids, liberated sugars, and NH_4^+ would then serve to support growth and further extracellular polymer synthesis. Polymer synthesis represents a BOD sink or removal mechanism when the flocs settle out. The effect is amplified because the polymers have been shown to adsorb dissolved organics, such as amino acids, prior to metabolism and would therefore remove more BOD than would be anticipated on the basis of metabolism. This latter effect is analogous to biosorption described by Eckenfelder (see McKinney, 65) and can explain the exceptional rate of BOD removal reported by Butterfield for these organisms.

The available data is consistent with the following viewpoint: Settled sludge of aerobic waste treatment processes is equivalent to the flocculent matrix described for laboratory cultures of microorganisms, which are composed of cells, extracellular polymers fibrils and any detrital or soluble compounds that adsorb to the fibrillar matrix. Under laboratory conditions the high molecular weight polymers adsorb large amounts of water and monovalent (M^+) cations (e.g. Na^+, K^+). Polyvalent cations can exchange with the bound water and M^+, thereby releasing water and the M^+ ions. The polymer then becomes more hydrophobic and flocculation results. The polymers are hydrolyzed under anaerobic conditions causing a release of adsorbed or complexed metal ion back into solution, which could be either recycled or discharged. Anaerobic digestion would severely penalize the objective of mineral nutrient removal and could contribute to algal growth and eutrophication if the discharge found its way to receiving water. Conversely, the aerobic process is quite effective in removing metal ions and would benefit receiving water by withholding algal mineral nutrients. A controlled process could be exploited as an advanced treatment procedure.

Flocculation in Natural Water

A large proportion of bacteria isolated from natural water are floc-formers. That is, they grow in an aggregated manner forming massive adherent particles (greater than 1.0 μm) rather than remain in suspension.

For example, 19 of 36 bacteria isolated from Lake Erie formed flocs in the laboratory in at least one growth medium. Relatively few have been examined using electron microscopic techniques to establish the presence of extracellular fibrils. However, all of those which have been examined in this manner do possess extracellular fibrils.

The interactions among floc-forming microorganisms, suspended and dissolved chemicals and other particulates (e.g. detritus and non-flocculent microbes) are similar to those observed in waste treatment systems. A greater variety of microorganisms will, however, be found in a lake environment as compared to the ecologically more homogeneous treatment systems. Some of these interactions have been described by Pfister et al. It is worth emphasizing that the adsorption of chemicals by floc-formers is a physical-chemical phenomenon related to chemical affinities and interactions with toxic substances and inert substances as well as with nutrients are to be expected.

Figures 46 through 50 illustrate some of the physical associations of microorganisms and other particulate material that result in a conglomerate type floc which is the Lake equivalent of activated sludge. The electron micrograph of _Z. ramigera_ I-16-M cells (C) presented in Fig. 46 represents an experimental illustration of how insoluble suspended magnesium silicate particles (P) can be adsorbed by extracellular polymer fibrils (F). These associations increase floc density and increase the ratio of inorganic to organic in sediment. Again, this results in a twofold adsorption effect because insoluble mineral particles are also known to adsorb chemicals from solution. Figure 47 is a photomicrograph showing individual bacterial cells (B) embedded in a capsular slime layer (S) which surrounds a clump of _Microcystis_ cells (M) taken from Lake Erie. Figure 48 is a fluorescent photomicrograph taken at lower magnification than Figure 6 and shows the association of both bacterial flocs (Bf) and inorganic silt particles (P) with filaments of unidentified green algae (GA). Figure 49 is a photograph of the identical field shown in Fig. 48 but taken under phase contrast illumination. The association of inorganic particles with algae and bacterial clumps (Bf) can be more easily seen when Fig. 8 is compared to Fig. 48.

Contemporary sediment which continuously forms and settles in Lake Erie was collected by Herdendorf using specially designed sediment collectors. This sediment is reported to have an average of about 10% organic and 90% inorganic content. Examination of the flocculent contemporary sediment shown in Fig. 50 after carbon replication and electron microscopy clearly shows bacterial cells (B), a diatom (Di), fibrillar material (F), and detrital material (De) which may either be organic or inorganic. As previously discussed, microbial material can be exceptionally high in mineral content and a 10% content of organics in sediments may be misleading in terms of its microbial activity.

Both floc-forming bacteria and detrital particles have been observed to adsorb chlorinated hydrocarbon pesticides in the laboratory and remove

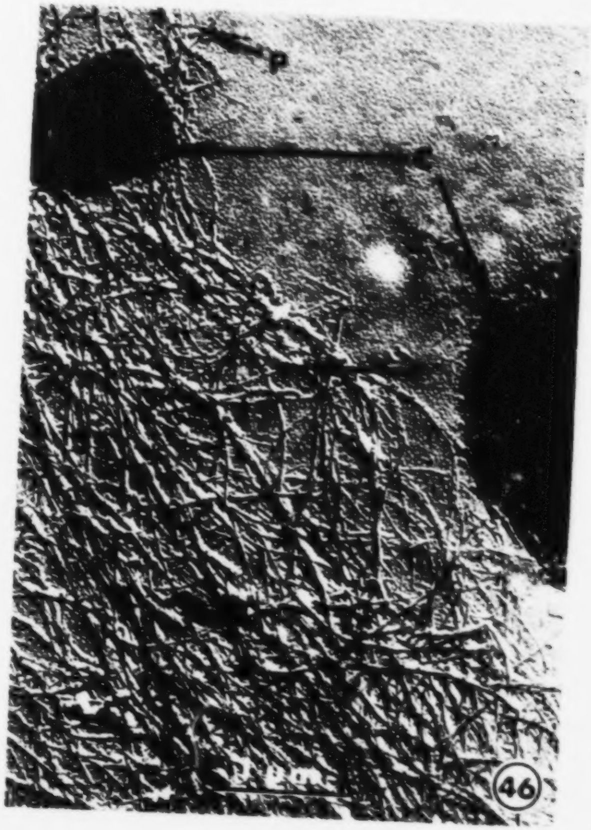


Figure 46. Electron micrograph of a shadow cast preparation of *Z. ramigera* I-16-M with insoluble particles (P) of magnesium silicate (talc) adsorbed to the extracellular polymer fibrils (F) and cells (C) X20,700

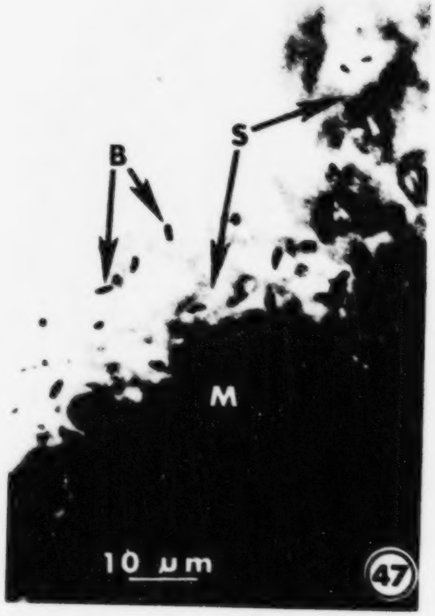


Figure 47. Photomicrograph of a wet mount of blue green alga *Microcystic* cells (M) containing bacteria (B) embedded within the extracellular slime layer (S). Cells were stained with dilute crystal violet to increase constrast of bacteria X1300

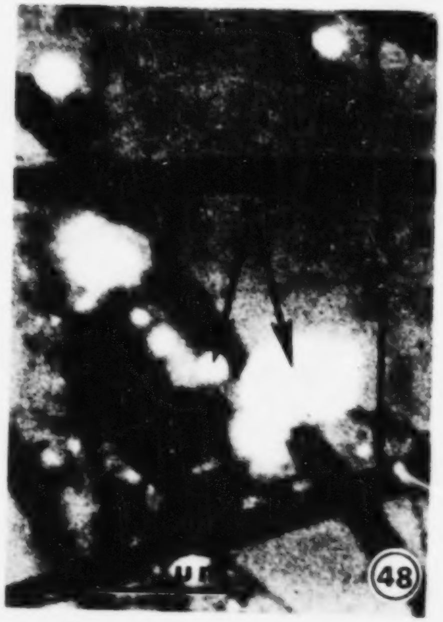


Figure 48. Photomicrograph of an unstained wet mount of naturally occurring algal-bacterial floc to which a suspension of insoluble fluorescent inorganic particles had been added. The photograph was taken under ultraviolet illumination and the highly fluorescent inorganic particles (P) can be seen in association with bacterial flocs (Bf) and green algal (GA) filaments (see Fig. 49) X380

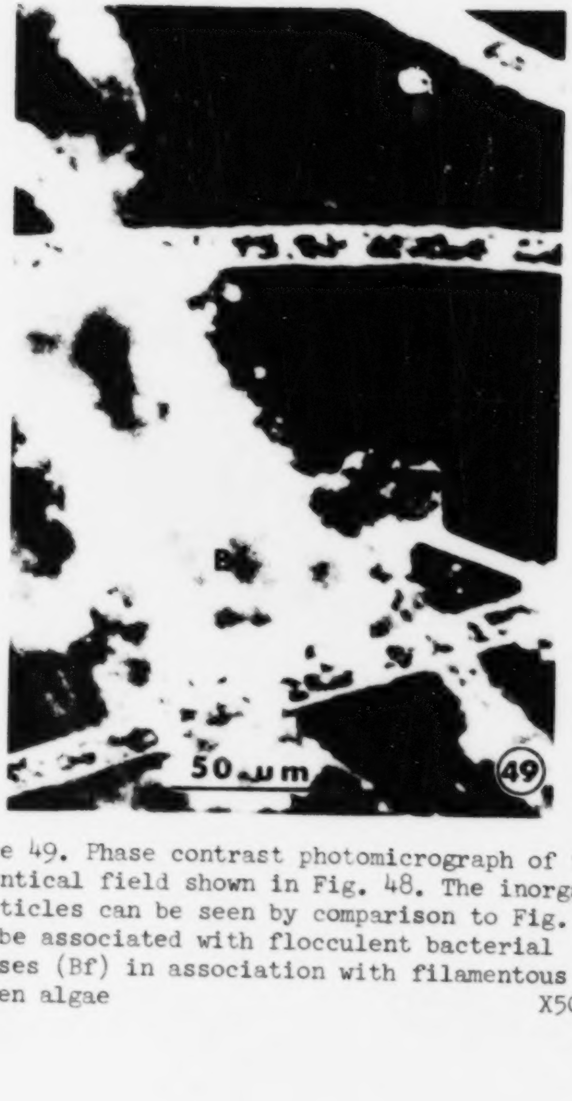


Figure 49. Phase contrast photomicrograph of the identical field shown in Fig. 48. The inorganic particles can be seen by comparison to Fig. 48 to be associated with flocculent bacterial masses (Bf) in association with filamentous green algae X500

them from suspension. The contemporary sediment shown in Fig. 50 also adsorbs chlorinated hydrocarbon pesticides and indeed contains a background content when obtained from Lake Erie. When the chemicals adsorb to floc and settle from the water-column, it represents a natural purification process. When the sediment accumulates on the bottom, it represents a BOD zone and a relatively high localized concentration of certain chemicals (e.g. pesticide). It is likely that the presence of high concentrations of pesticide in the sediment would exert an insecticidal effect on bottom insects and other fauna.

On the basis of laboratory and environmental observations, the generalized environmental scheme outlined in Fig. 51 can be postulated. Dissolved organic and inorganic nutrients enter the water-column (Lake) from a variety of sources including waste treatment effluents. Suspended solids including both organic detritus and inorganic particulates also enter from a variety of sources such as erosion and agricultural runoff. Floc-producing bacteria and algae synthesize polymer from dissolved organics which then adsorb more dissolved organics and inorganics, as well as suspended organic and inorganics, and other living microorganisms. When the resulting conglomerate reaches a critical mass and density (related to chemical and physical parameters) it settles from the water-column and accumulates on the bottom. This sediment is subject to either wind and wave action whereupon it is resuspended and recognized as increased water turbidity, or in deep zones it is subject to anaerobic hydrolytic activity and soluble components are released into the water-column to stimulate further ecological activity. Second order interactions always take place and the ecosystem has been over simplified. However, extracellular polymers produced by bacteria and algae probably play a significant role in the natural purification of water and in the eutrophication process.

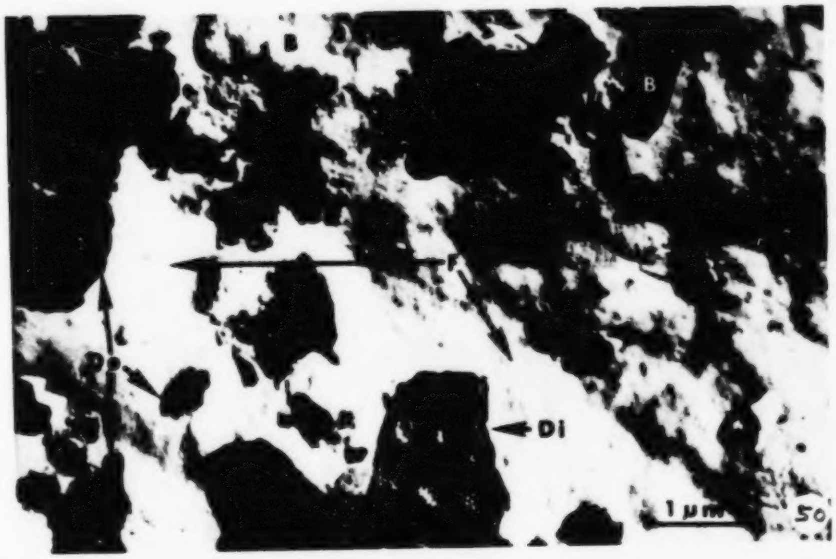

B
B
D
Di
1 µm
50

Figure 50. Electron micrograph of a carbon replica of contemporary sediment from Lake Erie

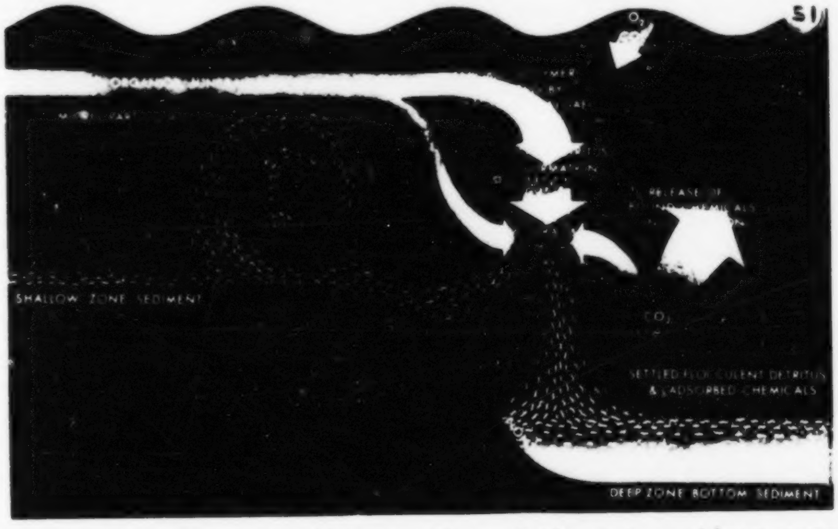

51
O_2
ORGANO-MINERAL
RELEASE OF BOUND CHEMICALS
SHALLOW ZONE SEDIMENT
CO_2
SETTLED FLOCCULENT DETRITUS & (ADSORBED) CHEMICALS
DEEP ZONE BOTTOM SEDIMENT

Figure 51. Schematic illustration of the interactions among dissolved organic and inorganic pollutants with suspended microparticles and polymers synthesized by microorganisms

SECTION VII

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SECTION VIII

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DISSERTATIONS AND THESIS

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Ph.D. Dissertations

Friedman, B. A., 1968. Identification of *Zoogloea* species and its exocellular matrix, and the relationship of this matrix to floc-formation and uptake of metallic ions.

Joyce, G. H., 1968. Biochemical studies of *Zoogloea ramigera* isolate 115 with emphasis on ethyl alcohol metabolism.

M.S. Thesis

Parsons, A. B., 1970. Investigations of *Zoogloea ramigera* isolate 115 with emphasis on composition and synthesis of the extracellular matrix polymer.

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